Effects of EHV-1 infection on the migration behavior of monocytic cells and on components of the basement membrane in the respiratory mucosa

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

aa	amino acids
ANOVA	analysis of variance
APC	antigen presenting cell
BM	basement membrane
CD	cluster of differentiation
CHO-K1	Chinese hamster ovary-K1 cell
CNS	central nervous system
CO2	carbon dioxide
CR	consensus repeats
CRD	completely randomized design
d	day
ds	double-stranded
DABCO	1,4-diazobicylo-2.2.2-octane
DC	dendritic cell
DMEM	dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
E	early
ECM	extracellular matrix
EHM	equine herpes myeloencephalopathy
EHV-1	equine herpesvirus type 1
FACIT	Fibril-associated collagen with interrupted triple helix
FITC	fluorescein isothiocyanate
FITC-OVA	FITC conjugated ovalbumin
GMP-140	granule membrane protein 140
h	hour
HCMV	human cytomegalovirus
HEV	high endothelial venule
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
ICAM-1	intercellular adhesion molecule-1
iDC	immature dendritic cell
IE	immediate early
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
Kbp	kilo base pairs
kDa	kilo Dalton
L	late
LFA-1	lymphocyte function-associated antigen-1

LN	lymph node
mAbs	monoclonal antibodies
Mac-1	macrophage-1 antigen
MACS	magnetic activated cell sorting
MAdCAM-1	mucosal vascular addressin cell adhesion molecule-1
MALT	mucosa associated lymphoid tissue
mDC	mature dendritic cell
MFI	mean fluorescence intensity
MHC I	major histocompatibility complex class I
MHC II	major histocompatibility complex class II
NALT	nasal associated lymphoid tissue
ORF	open reading frame
PADGEM	platelet activation-dependent granule to external membrane protein
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PECAM-1	platelet endothelial cell adhesion molecule-1
PRV	pseudorabies virus
PSGL-1	P-selectin glycoprotein ligand 1
RBCs	red blood cells
RK	rabbit kidney
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RSD	arginine, serine, and aspartic acid
SD	standard deviation
SHV-1	suid herpesvirus 1
SPSS	statistical package for the social sciences
SSC	side scatter
TACE	tumor necrosis factor-alpha converting enzyme
TAP	transporter associated with antigen processing
TCID50	tissue culture infectious dose with a 50% endpoint
TEM	transendothelial migration
U_L	unique long
URT	upper respiratory tract
Us	unique short
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
VZV	varicella zoster virus
vWF	von Willebrand factor
WBCs	white blood cells

CHAPTER I

INTRODUCTION

1. Phylogenetic background

1.1. *Herpesvirales*

In 2009, the International Committee on Taxonomy of Viruses (ICTV) assigned a new order, *Herpesvirales* (Davison et al., 2009). The order *Herpesvirales* is comprised of large DNA viruses that infect a wide variety of animals, from mammals to bony fish and molluscs. This order contains three distinct families: the *Herpesviridae* that includes herpesviruses of mammals, birds, and reptiles; the *Alloherpesviridae* that includes the herpesviruses of fish and frogs, and the *Malacoherpesviridae* that contains a virus of oysters (bivalve) (Wolf and Darlington, 1971; Davison et al., 2005; Davison et al., 2009).

The *Herpesviridae* family is further subdivided into three distinct subfamilies, alpha (α)- , beta (β)- and gamma (γ)- herpesviruses based on their host range, clinical symptoms, disease severity, tissue tropism and replication kinetics (Roizman and Baines, 1991; Roizman et al., 2001). *Alphaherpesvirinae* and *Betaherpesvirinae* can be considered as 'lytic' because they cause a lytic replication in a broad array of cells. However, in specific cell types they may stay lifelong in a latent stage (i.e. neurons). *Gammaherpesvirinae* replicate in a narrow range of cells and often remain latent and persistent in cells (Ackermann, 2006). The subfamilies are subdivided into genera, based on similarities in genome sequence arrangement, DNA sequence homology and relatedness of important viral proteins. The phylogenetic tree of the *Herpesvirales* is shown in Figure 1.

1.2. *Alphaherpesvirinae*

In general, *Alphaherpesviruses* are recognized to have a broad host cell range with a rapid replication cycle, followed by destruction of the host cell in a wide variety of susceptible cells and swift spread among these cells. They use multiple strategies to hijack infected host immune cells, establish latent infection and evade antiviral immune responses in order to eventually permit the production and subsequent dissemination of infectious virions (Fields et al., 2007; Steukers et al., 2012). Based on sequence analysis and molecular criteria, the *Alphaherpesvirinae* are subdivided into four genera: *Simplexvirus, Varicellovirus, Mardivirus and Iltovirus.* The members of the *Simplexvirus* genus are human herpesvirus 1 (herpes simplex type 1; HSV-1), human herpesvirus

2 (herpes simplex type 2; HSV-2), herpesvirus B (HVB), bovine herpesvirus 2 (BoHV-2), herpesvirus saimiri 1 (HVS-1) and simian agent 8 (SA8). Equine herpesvirus 1 (EHV-1) has been classified to the *Varicellovirus* genus together with pseudorabies virus (PRV), varicella zoster virus (VZV), bovine herpesvirus 1 (BoHV-1), feline herpesvirus 1 (FHV-1), equine herpesvirus 3 (EHV-3) and equine herpesvirus 4 (EHV-4). Marek's disease virus 1 (MDV-1), Marek's disease virus 2 (MDV-2) and herpesvirus of turkeys (HVT) belong to the *Mardivirus* genus and infectious laryngotracheitis virus (ILTV) belongs to the *Iltovirus* genus (Roizman and Baines, 1991; Tischer and Osterrieder, 2010; Zaichick et al., 2011).

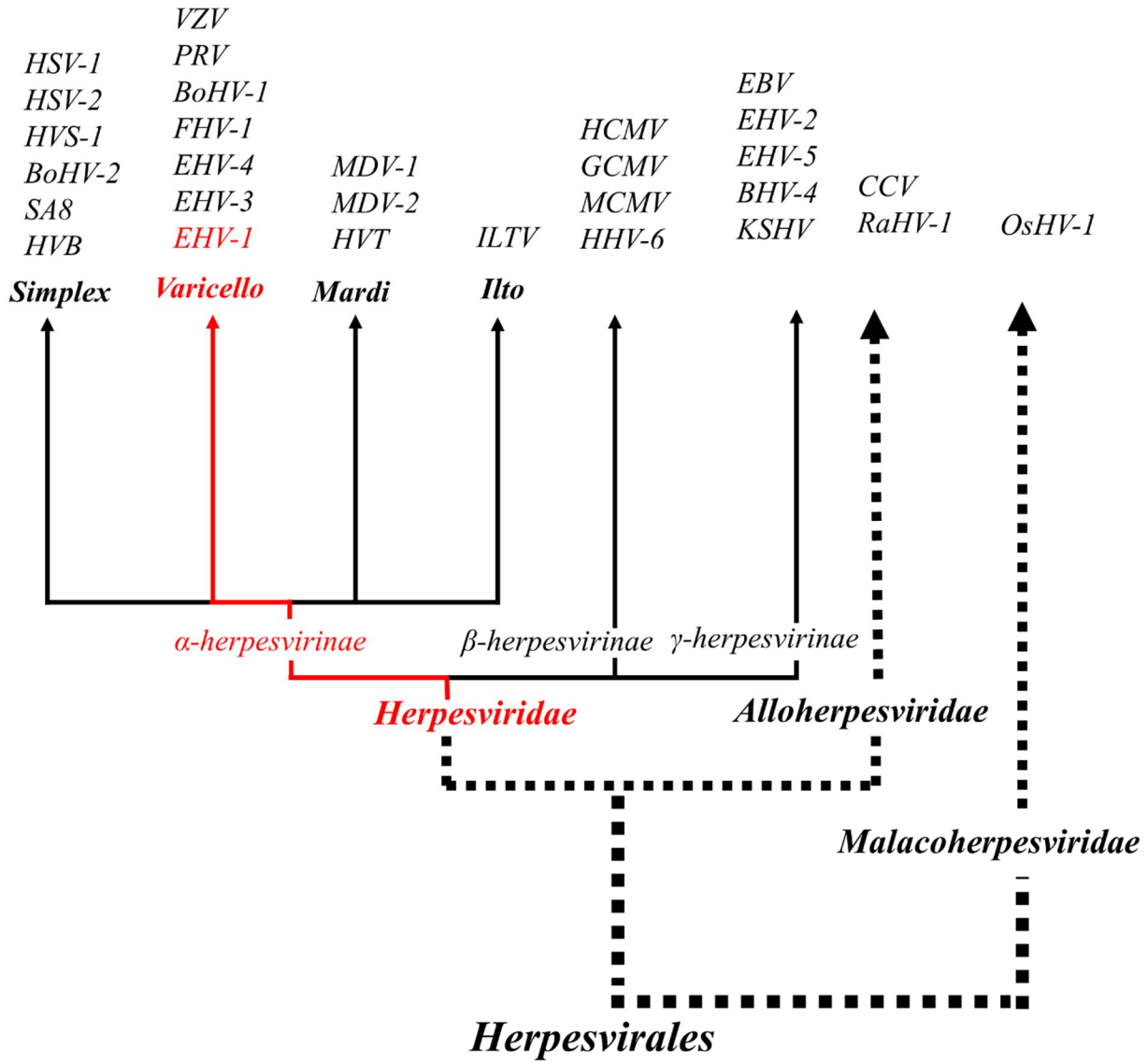

Figure 1. *Herpesvirales phylogenetic tree (adapted from Davison, 2002).*

2. Equine herpesvirus type 1

2.1. Virus structure

There are four morphologically distinct components which make up the equine herpesvirus type 1 (EHV-1) virion: the viral genome, the capsid, the tegument and the envelope. The linear double stranded DNA genome of approximately 150 kbp is enclosed in an icosadeltahedral capsid (approximately 125 nm diameter), which is surrounded by a tegument, proteinaceous matrix. The tegument is encoded by at least 15 genes and contains many virus-coded proteins. The tegument protein is encompassed by a lipid envelope, containing membrane-associated proteins (Fields et al., 2007; Roizman and Knipe, 2001; Roizman and Pellett, 2001; Davison et al., 2009).
The general structure of an EHV-1 virion is given in Figure 2.

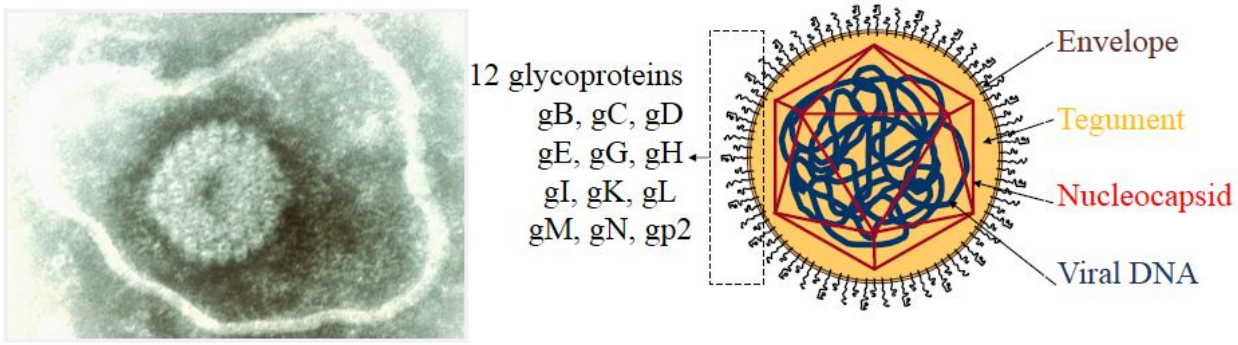

***Figure 2**. Structure of an EHV-1 virion (adapted from Paillot et al., 2008).*

Alphaherpesvirinae glycoproteins are named according to the nomenclature established for the prototype member of the subfamily, herpes simplex virus type 1 (HSV-1). Homologues of glycoproteins gB, gC, gD, gE, gG, gH, gI, gK, gL, gM, gN have been identified in the EHV-1 genome. Viral glycoprotein gp2 is unique to EHV-1. Envelope glycoproteins have three parts: the external ectodomain, a transmembrane segment, and the internal part, the endodomain. The exact locations of the EHV-1 glycoprotein genes in the genome have been determined by sequence analysis. These 12 envelope glycoproteins are involved in virus attachment, penetration, egress, cell-to-cell spread, pathogenicity and virulence (Turtinen and Allen, 1982; Osterrieder and Van de Walle, 2010).

Glycoprotein B (gB)

Envelope glycoprotein B is highly conserved throughout the *Herpesviridae* family and presumably has a function in attachment and membrane fusion (Eisenberg et al., 2012; Li et al., 2006). EHV-1 gB plays a pivotal role in penetration and in direct cell-to-cell spread of virions (Neubauer et al., 1997, Wellington et al., 1996b). Perturbations of the carboxyl terminal domains of gB facilitate gB-mediated cell-to-cell fusion, however the absolute requirement of this glycoprotein in the fusion process in EHV-1 is still somewhat ambiguous (Neubauer et al., 1997). Glycoprotein B along with major nucleocapsid proteins are some of the most antigenic EHV-1 proteins (Ahmed et al., 1993).

Glycoprotein C (gC)

EHV-1 gC binds to heparan sulfate (HS) molecules, a heparin-like moiety on the cell surface, and initiates cell entrance of the virus (Sugahara et al., 1997; Osterrieder, 1999; Azab et al., 2010). Hydrophilic regions of the gC may be responsible for binding to heparin (Sugahara et al., 1997). Glycoprotein C is capable to bind the third component of complement (C3), which allows the virus to escape from complement-mediated inactivation of cell-free viruses and complement-mediated lysis of infected cells (Azab et al., 2010; Huemer et al., 1995). EHV-1 gC also plays a major role in the release of virions (Osterrieder, 1999).

Glycoprotein D (gD)

The EHV-1 envelope glycoprotein D is encoded by a unique short (Us) segment of the EHV-1 genome with only 26% and 20% of its residues matching PRV gD and HSV-1 gD, respectively, and consists of 385aa (Audonnet et al., 1990; Elton et al., 1992; Flowers and O'Callaghan, 1992; Love et al., 1992; Whalley et al., 1991). gD is conserved among almost all alphaherpesviruses (Connolly et al., 2011). EHV-1 gD is required for virus entry, which occurs via cell-cell fusion and/or endocytosis (Csellner et al., 2000; Van de Walle et al., 2008; Azab and Osterrieder, 2012). As shown in Table 1, the cell type and the respective receptors are the main determinants of whether the fusion process occurs at the plasma membrane or through an endocytic route of entry. Previous studies have proven that the C-terminal region of HSV-1 gD (encompassing residues 260 to 310) is required to trigger fusion (Lazear et al., 2008; Connolly et al., 2011). This mechanism of gD triggering fusion is shared among alphaherpesviruses (Geraghty et al., 1998). Glycoprotein

Table 1. *Entry of EHV-1 into different cell types.*

Cell type	Entry pathway(s)	Receptor(s)	References
equine PBMC	endocytosis	αV integrins	Van de Walle et al., 2008; Azab and Osterrieder, 2012
equine endothelial cells	fusion at the plasma membrane (endocytosis at low efficiency)	MHC-I	Van de Walle et al., 2008; Azab et al., 2014; Kurtz et al., 2010
equine dermal cells	fusion at the plasma membrane	MHC-I	Frampton et al., 2007; Sasaki et al., 2011
RK13 cells	fusion at the plasma membrane	MHC-I	Frampton et al., 2007
CHO-K1 cells	endocytosis	αV integrins, glycosaminoglycans	Frampton et al., 2005: Van de Walle et al., 2008

D is the key receptor-binding protein of numerous alphaherpesviruses (Campadelli-Fiume et al., 2007). In general, the gD receptors include members of the tumor necrosis factor (TNF) receptor family (HveA), the poliovirus receptor family (HveB and HveC, members of the immunoglobulin superfamily), integrins, and a modified form of heparan sulfate called 3-O-sulfated heparan sulfate. The cells that express integrin alpha V, HveA, HveB, HveC, and heparan sulfate can be infected by EHV-1 (Frampton et al., 2005; Azab and Osterrieder, 2012). For EHV-1, Kurtz et al. (2010) showed that the major histocompatibility complex class I (MHC-I) molecules play a role as a gD receptor for EHV-1 entry into equine cells. Recently, Azab et al. proved that EHV-1 and EHV-4 target classical polymorphic MHC-I molecules as viral entry receptors (Azab et al., 2014). The entry of some alphaherpesviruses through an endocytic route was described for certain cell types, such as HeLa and Chinese hamster ovary (CHO-K1) cells, which depend on binding of gD to its receptor, integrin alpha V (Nicola and Straus, 2004; Nicola et al., 2005). Entry of EHV-1 has also been suggested to occur via an endocytic pathway to infect important cell populations. The interaction between cellular integrins and an arginine, serine, and aspartic acid (RSD) motif of EHV-1 envelope gD plays a critical role for virus entry via endocytosis (Van de Walle et al., 2008).

Monoclonal antibodies against gD can neutralize EHV-1 infectivity and hamper virus peneteration into the cells (Csellner et al., 2000; Van de Walle et al., 2008).

Glycoprotein E (gE)

In general, glycoprotein E together with glycoprotein I (complex gE/gI) forms a non-covalently bound heterodimer that functions in viral cell-to-cell spread and transsynaptic spread of infection throughout the host nervous system (Favoreel et al., 1999). gE is a non-essential glycoprotein, except for Marek's disease virus serotype 1 and varicella-zoster virus (Mo et al., 2002; Schumacher et al., 2001). Although the gE/gI complex is involved in cell-to-cell spread of EHV-1, the complex is not involved in the process of virus maturation and release or in virus attachment and penetration (Matsumura et al., 1998). gE is known as a virulence factor of EHV-1 in horses and intramuscular vaccination with an attenuated gE deletion mutant provided foals with partial protection against EHV-1 respiratory disease (Tsujimurai et al., 2009).

Glycoprotein G (gG)

Glycoprotein G is a non-essential protein of EHV-1 that can be found in three distinct isoforms: a full-length membrane-bound form, a smaller membrane-bound form lacking the majority of the extracellular domain, and an important secreted form that is made up of the extracellular domain released by cleavage from the membrane anchor (von Einem et al., 2007; Osterrieder and Van de Walle, 2010). Both the secreted form and membrane-bound form of EHV-1 gG bind chemokines (Osterrieder and Van de Walle, 2010; Van de Walle et al., 2008). Therefore, gG acts as a viral chemokine binding protein (vCKBP), by blocking interaction of chemokines with both chemokine specific receptors and glycosaminoglycans (Thormann et al., 2012). It has been shown that the hypervariable region of EHV-1 gG is responsible for chemokine binding and function (Van de Walle et al., 2009). One of the notable functions of gG is interfering with leukocyte migration in tissue which can contribute to virulence and organ dissemination of EHV-1 (Osterrieder and Van de Walle, 2010). By performing chemotaxis assays, it was shown that gG of EHV-1 is able to hinder transmigration of leukocytes in response to recombinant equine interleukin 8 (IL8, also designated CXCL8) (Van de Walle et al., 2007; Thormann et al., 2012).

Glycoprotein H (gH)

Glycoprotein H forms a heterodimer with glycoprotein L (complex gH/gL) and this complex acts as a fusion regulator for many herpesviruses (Eisenberg et al., 2012; Azab et al., 2012). Analysis of the gH structure revealed that it has three distinct domains in the N-terminal region (domain H1) that bind to gL (Chowdary et al., 2010). In the absence of gL, expression of gH polypeptide in transfected cells is not folded and processed correctly. Recently, it has been shown that EHV-1 gH and cellular α4β1 integrins are important determinants in the choice of virus cellular entry pathways (Azab et al., 2013).

Glycoprotein I (gI)

Glycoprotein I forms a non-covalently bound heterodimer with gE (complex gI/gE) which facilitates cell-to-cell spread of viruses and is one of the important factors of EHV-1 virulence (Matsumura et al., 1998). Yet, some aspects of the EHV-1 gI structure and function are still unclear and need to be elucidated.

Glycoprotein K (gK)

The gK of herpesviruses is present in the virions and also on the plasma membrane of cells that are infected with the virus. The EHV-1 gK is required for efficient cell-to-cell spread and virus egress (Neubauer and Osterrieder, 2004). Immediate early (IE) phosphoprotein of EHV-1 binds to the transcription initiation site of the gK promoter sequences, thereby repressing transcription of this true late gene (Kim et al., 1999; Derbigny et al., 2000).

Glycoprotein L (gL)

Glycoprotein L is associated with gH, however the specific role of the gH/gL complex is the least well understood in the herpesvirus family. gL is an essential glycoprotein in some herpesviruses, but its function and role in the EHV-1 replication cycle remains largely unknown (Stokes et al., 1996; Roop et al., 1993). A properly formed gH/gL heterodimer is needed for attachment, virus entry, and virus-induced cell-to-cell fusion of alphaherpesviruses (Eisenberg et al., 2012). In spite of the fact that alphaherpesviruses lacking gH/gL are unable to enter cells, they are able to attach to the cell surface. Therefore, the role of the gH/gL in virus entry is important during the virus

envelope plasma membrane fusion event and is not required for virion or receptor binding (Eisenberg et al., 2012).

Glycoprotein M (gM)

The conserved but nonessential gM glycoprotein represents a hydrophobic class III membrane protein that contains eight putative transmembrane domains and form a complex with gN (pUL49.5) (Osterrieder et al., 1996; Jöns et al., 1998; Crump et al., 2004). Processing of gM inside the EHV-1-infected cell depends on the expression of gN (Rudolph et al., 2002). EHV-1 gM is associated with not only the entry of the virus but also the direct spread from cell to cell (Osterrieder et al., 1996). Deletion of gM in strain RacL11 of EHV-1 leads to a 50% reduction in plaque size compared to the wild type, and a 50 to 100 times less production of extracellular virus (Osterrieder et al., 1996; Osterrieder et al., 1997).

Glycoprotein N (gN)

The function of gN is not well characterized in EHV-1. Rudolph et al have shown that gN is necessary and sufficient for functional processing of glycoprotein M, and that it is required for efficient virus replication (Rudolph et al., 2002). Alphaherpesviruses evade elimination by cytotoxic T lymphocytes through specific interfering with the transporter associated with antigen processing (TAP) and subsequent antigen-presenting function of major histocompatibility complex class I (MHC I) and in this context, gN has been shown to act as a TAP inhibiting molecule (Verweij et al., 2011).

Glycoprotein 2 (gp2)

The unique mucin-like high molecular mass (Mr) glycoprotein 2 (gp2) has only been recognized in the *equid alphaherpesviruses*. The gp2 (also known as gp300) is a large (250-kDa) glycoprotein of EHV-1 and is encoded by gene 71, located within the unique short region of the genome, with abundant O-glycosylation sites in the N-terminal part of the molecule (Smith et al., 2005; Wellington et al., 1996a; Hansen et al., 1995). gp2 is a major EHV-1 virulence factor. Mutation of gene 71 attenuated the virus but did not inhibit virus replication in the murine lung (Smith et al., 2005; Frampton et al., 2002). Deletion of gp2 impairs virus cell-to-cell spread and secondary envelopment (Rudolph et al., 2002; Sun et al., 1996). Smith and colleagues suggest that gp2 act as

a signaling molecule and this function is being explored by generating mutant viruses (strain KyARgp2F) that lack the ability to release the secreted form of the protein (Smith et al., 2005).Taken together, this envelope glycoprotein plays an important role in EHV-1 pathogenesis.

2.2. Virus genome and replication cycle

Genomic organization

The complete genome sequence of EHV-1, with 80 open reading frames (ORFs), has been elucidated in 1992 by Telford et al. (Telford et al., 1992). The genome of the virus is organized as a linear double-stranded type D DNA of approximately 150kbp with a base composition of 56 to 57 % G + C (Telford et al., 1992). Since four ORFs are duplicated, the genome is considered to contain at least 76 distinct genes with a potential to code for 77 different proteins due to alternative splicing of ORF64 (Telford et al., 1992; Harty et al., 1989). The EHV-1 genome is divided into 2 covalently linked components: the S or short component that consists of a unique (US) sequence and the L or long region that comprises a unique (UL) sequence, with the latter bracketed by two inverted repeat regions, namely the internal repeat (IR) and terminal repeat (TR), Figure 3, (Telford et al., 1992; Yalamanchili and O'Callaghan, 1990).

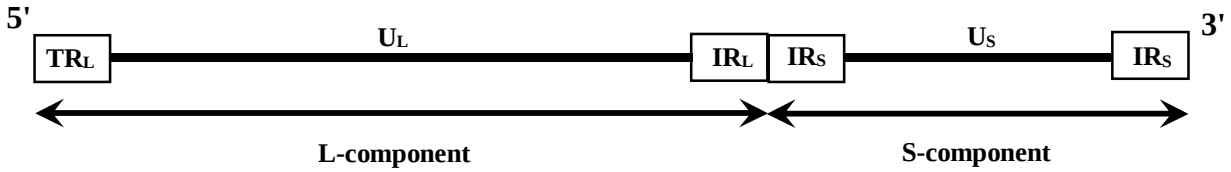

Figure 3. *Genomic organization of EHV-1. TR, terminal repeat; IR, internal repeat; U, unique.*

Replication cycle

A schematic representation of the various steps of the EHV-1 replication cycle is shown in Figure 4.

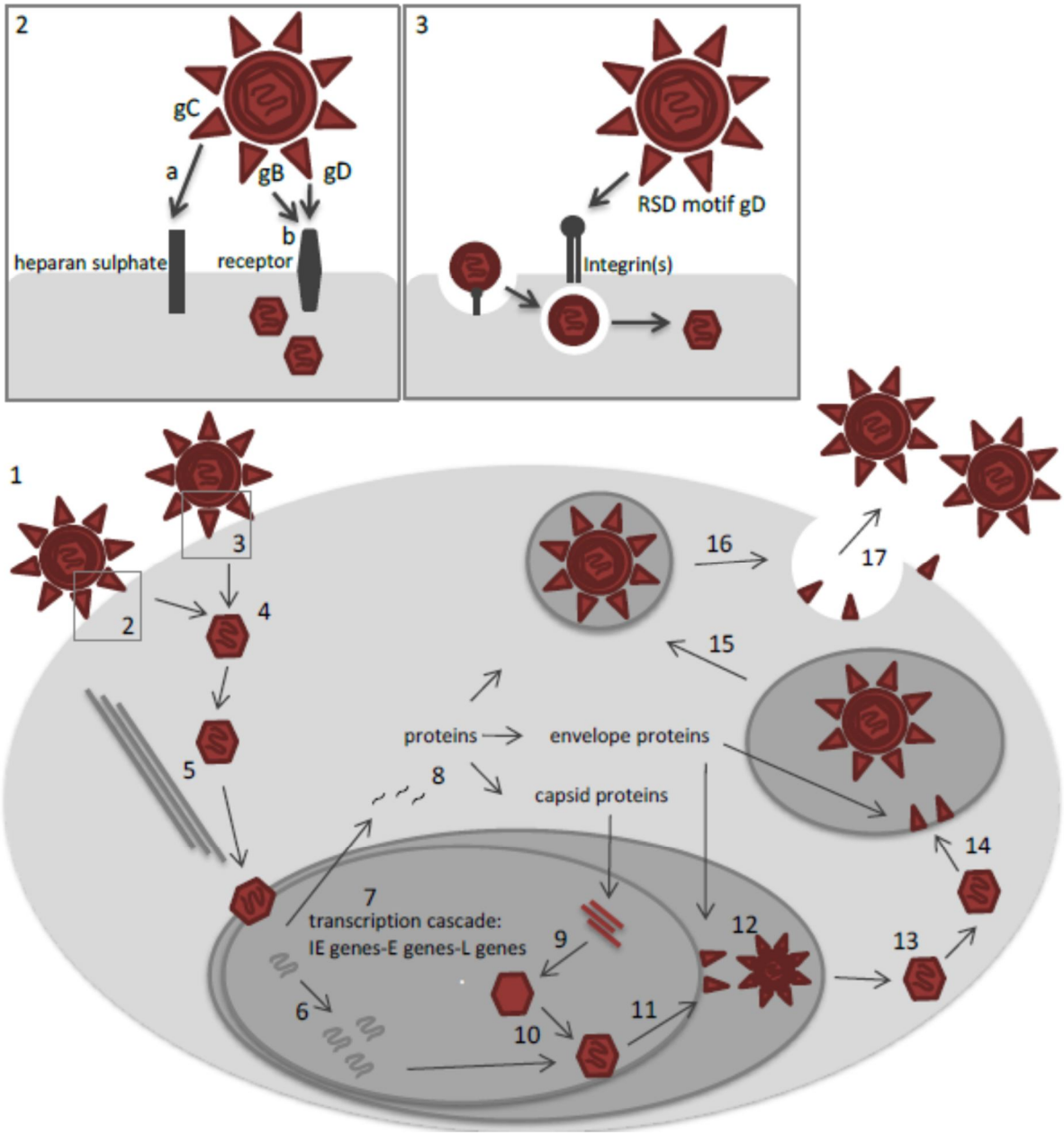

Figure 4. *The EHV-1 replication cycle (Vandekerckhove, 2011). The first step in the replication cycle is the attachment of free virions to the surface of the target cell (1). The binding is initially unstable and includes interaction of viral envelope glycoproteins gB and gC with heparan sulfate glycosaminoglycan moieties of the cell surface (2a). Subsequently, EHV-1 can enter cells via 2 pathways, depending on the infected cell type. In equine endothelial, equine dermal and rabbit kidney cells, fusion of the viral envelope with the plasma membrane occurs through interaction of gD with an entry receptor, possibly MHC-1 (2b). In equine PBMCs and CHO-K1 cells, entry occurs via a nonclassical endocytic pathway mediated by the interaction between cellular αV*

integrins and an RSD motif at amino acid (aa) positions 152-154 present in EHV-1 gD (3). The nucleocapsid is released into the cytoplasm (4) and transported to the nucleus along microtubules (5). In the nucleus, DNA replication (6) and transcription (7) occur. The genome is transcribed in a cascade-like manner with first the immediate-early (IE) genes, then the early (E) genes and finally the late (L) genes. RNA molecules are transported to the cytoplasm and translated to proteins (8). Capsid proteins are redirected into the nucleus where assembly of the capsid occurs (9). Subsequently, DNA is pulled into the newly formed capsids and hence nucleocapsids are assembled (10). The nucleocapsids leave the nucleus via budding through the inner leaflet of the nuclear membrane (11), hereby requiring their primary envelope (12). Subsequent fusion with the outer leaflet of the nuclear membrane results in the entry of naked nucleocapsids into the cytoplasm (13). The nucleocapsids acquire their secondary envelope at the Golgi apparatus (14) and then leave the cell via vesicle-mediated exocytosis (15, 16, 17).

The replication cycle of EHV-1, and alphaherpesviruses in general, begins with the attachment of free virions to the surface of a target cell, a process in which at least five viral glycoproteins are involved: gB, gC, gD and the gH-gL complex (Spear and Longnecker, 2003). Therefore, the cycle starts when viral gC undergoes transient interactions with cellular surface receptors (Kurtz et al., 2010; Sasaki et al., 2011). Glycoprotein D establishes a more stable attachment, binding to one of its receptors (Geraghty et al., 1998; Granzow et al., 2005). While entry receptors for several alphaherpesviruses, such as herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), pseudorabies virus (PRV) and bovine herpesvirus 1 (BoHV-1), have already been identified, studies on entry receptors for EHV-1 are ongoing. Some of these receptors are listed in Table 1. Following this interaction, the viral envelope is fused with the target cell membrane with the concerted action of the viral fusion protein gB and complex gH-gL, releasing the particle into the cytoplasm of the target cell (Spear and Longnecker, 2003). Entry of EHV-1 into the cells happens by direct fusion at the plasma membrane or after endocytic uptake (Azab et al., 2013; Frampton et al., 2007; Hasebe et al., 2009; Van de Walle et al., 2008). After nucleocapsid release in the cytoplasm, capsids misuse the cellular motor protein dynein for direct microtubule-based transport towards the nuclear pore complexes. At the nuclear pores, docking complexes are formed and the viral DNA is released within the nucleus (Sodeik et al., 1997; Zaichick et al., 2011). In the infected nucleus, a cascade of transcription drives several phases of the viral replication cycle. Viral genes

can be subdivided into three categories and are sequentially transcribed: immediate-early (IE), early (E) and late (L) genes (Nicoll et al., 2012). First, the immediate-early (IE) gene is transcribed which results in the production of a regulatory protein which is pivotal for activating transcription of early and late genes. Early genes encode the proteins involved in virus replication and late genes encode the viral structural proteins. The IE transcription is orchestrated by a transactivator protein, brought into the cell as a tegument protein, namely VP16 (LaBoissiere and O'Hare, 2000). This tegument protein, VP16, transactivates the transcription of the only immediate-early protein of EHV-1, which is encoded by ORF64 (the homologue to ICP4 of HSV-1) and transcribed by cellular RNA polymerase II (Paillot et al., 2008). The early (E) gene products (UL5, UL8, UL9, UL29, UL30, UL42 and UL52 gene products) mainly play a role in nucleotide metabolism and DNA synthesis (Lehman and Boehmer, 1999). The transcriptional cascade continues with structural proteins, enzymes involved in assembly, and membrane proteins. UL31 and UL34 viral proteins are required in the assembly of all herpesviruses (Mettenleiter et al., 2006). With the involvement of these proteins and US3 protein kinases, nucleocapsids bud through the inner nuclear membrane thereby achieving tegument proteins and a primary envelope. Primary enveloped nucleocapsids, located in the perinuclear space, will undergo a subsequent fusion with the outer nuclear membrane. Capsids subsequently obtain their secondary, mature envelope at vesicles derived from the trans-Golgi network. Then, the mature virions are transported to the cell surface within sorting vesicles and released by fusion of glycoprotein-containing Golgi derived vesicles with the plasma membrane (Mettenleiter et al., 2009; Mettenleiter et al., 2006; Pomeranz et al., 2005).

2.3. Pathogenesis of EHV-1

EHV-1 is highly contagious. Transmission of the virus occurs by direct contact with infected saliva or nasal discharge. Although to a lesser extent, EHV-1 can be transmitted by aerosol or fomites (feed, clothing, boots, hands, etc.) (Allen and Bryans, 1986). Among the nine equine herpesviruses identified up till now, EHV-1 is one of the most pathogenic herpesviruses of horses. An overview of the complete pathogenesis of EHV-1 is given in Figure 5.

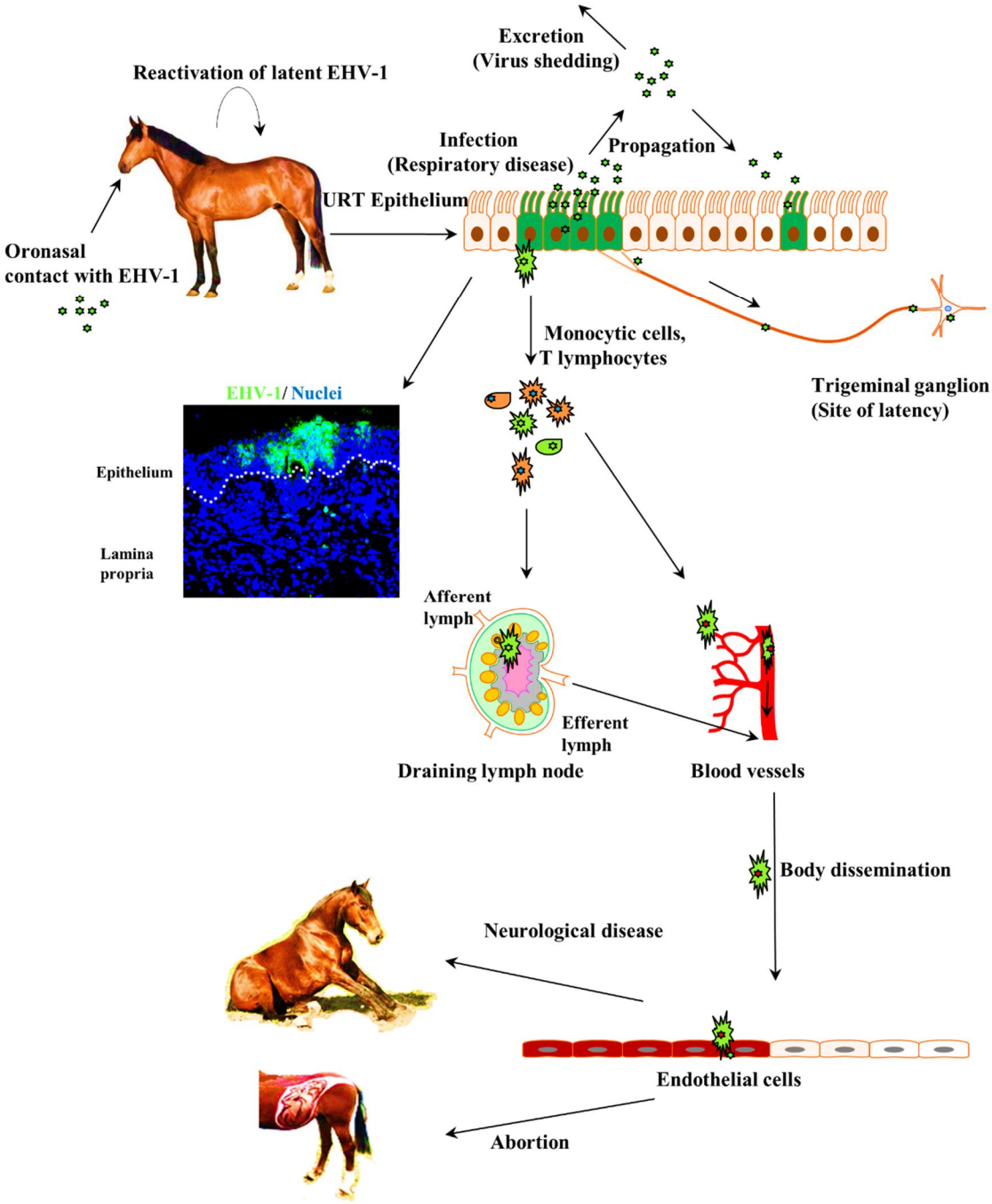

Figure 5. *Schematic overview of the pathogenesis of EHV-1. After inhalation or oral uptake, the virus replicates in the epithelium of the upper respiratory tract (URT). Then, by infecting monocytic cells and T lymphocytes it reaches lymph nodes and blood vessels. Replication of EHV-1 in the endothelial cells of the vessels of the pregnant uterus or the CNS induces abortion or neurological disease. EHV-1 can establish latency after primary infection, from which it can be reactivated and may cause diseases*

Infection of the respiratory epithelium

After inhalation or oral uptake of EHV-1 by the horse, the virus replicates in the epithelial cells of mainly the upper respiratory tract resulting in viral shedding and distinct herpetic lesions (Kydd et al., 1994; Patel and Heldens, 2005). The infected upper respiratory tract includes nasal septum, turbinates, nasopharynx, soft palate and trachea (Van Maanen, 2002; Gryspeerdt et al., 2010; Vandekerckhove et al., 2010; Allen et al., 1994). In the stage of EHV-1 replication in the upper respiratory tract, large amounts of virus are shed from the respiratory mucosa of infected animals into the external environment. Viral shedding generally starts at 1 day post infection and can last for 7 to 14 days post infection (Gryspeerdt et al., 2010; Heldens et al., 2001). Infection of the lower respiratory tract including epithelial cells, endothelial cells and leukocytes of lungs can be observed from day 2 to day 13 post viral infection, with a peak on day 9 when occasional non-occlusive thrombi may occur in the pulmonary interstitium (Kydd et al., 1994). The majority of respiratory infections cause a subclinical course. However, animals which are severely affected show vasculitis, haemorrhages and edema in the lungs and may die suddenly during a period of respiratory distress. The airway form of EHV1 infection is referred to as rhinopneumonitis and is characterized by rhinopharyngitis and tracheobronchitis (del Piero and Wilkins, 2001; del Piero et al., 2000).

Replication of EHV-1 in the mucosal epithelium of the URT happens in a plaque-wise manner. Plaques do not breach the basement membrane barrier. Instead, individual infected leukocytes (mainly monocytic cells and T lymphocytes) are hijacked by the virus to cross the basement membrane barrier en route to the blood vessels of the lamina propria and the draining lymph nodes (Van Maanen, 2002; Gryspeerdt et al., 2010; Vandekerckhove et al., 2010).

Cell-associated viremia

After a first replication in the respiratory epithelial cells, EHV-1 disseminates through the basement membrane (BM) barrier by single infected mononuclear immune cells, en route to blood vessels and draining lymph nodes. This results in a cell-associated viremia lasting for up to 8 to 18 days post infection (Kydd et al., 1996; Gryspeerdt et al., 2010; Edington et al., 1986; Gibson et al., 1992). The infected cells are identified as predominantly monocytic cells and T-lymphocytes (Table 2) (Gryspeerdt et al., 2010; Vandekerckhove et al., 2010). During viremia, the majority of

infected mononuclear immune cells do not show viral envelope proteins on their surface (van der Meulen et al., 2006). It has been well documented that peripheral blood mononuclear cells (PBMCs) are susceptible to EHV-1 infection in both *in vitro* and *in vivo* studies (Kydd et al., 1994; van Der Meulen et al., 2000; Gryspeerdt et al., 2010; Vandekerckhove et al., 2010; Wilsterman et al., 2011; Goodman et al., 2007; Van de Walle et al., 2009). However, which PBMC subpopulations are infected with EHV-1, and the load of virus in each cell subpopulation is a matter of debate. It can be said that the majority of EHV-1-infected cells are of the monocytic lineage, whereas T-lymphocytes can be considered as the second most important infected cells. Therefore, these different immune cell populations will be discussed here in detail.

Table 2. *Identification of EHV-1-infected leukocytes in nasal mucosa and PBMC upon in vivo, ex vivo and in vitro inoculation.*

Tissue/Cells	Condition	% of EHV-1-infected cells in a population of mononuclear cells	% of mononuclear cells in a population of EHV-1-infected cells	References
nasal mucosa	*in vivo*		monocytic cells: 53.4 ± 22.8 % T-cells: 16.2 ± 11.7 % B-cells: 0.3 ± 0.8 %	Gryspeerdt et al., 2010
	ex vivo	monocytic cells: 11.5 ± 4.5 %	monocytic cells: 74.1 ± 7.3 % T-cells: $CD4^+$: 14.3 ± 7.4 % $CD5^+$: 12.5 ± 4.4 % $CD8^+$: 4.9 ± 1.6 % B-cells: 0.0 ± 0.0 %	Vandekerckhove et al., 2010; Bannazadeh Baghi and Nauwynck, 2014
PBMC	*in vitro*	monocytic cells: 7.5 ± 4.4 % T&B-lymphocytes: 0.9 ± 0.6 %	monocytes: > 60% T-cells: $CD4^+$: 1.4 ± 1.6 % $CD8^+$: 3.7 ± 4.7 % B-cells: > 20 %	Van der Meulen et al., 2000; Yeo et al., 2013; Ma et al., 2010
	in vivo	mononuclear cells: 0.0004 %	monocytic cells: 78.5 % T-cells: $CD5^+$: 21.5 %	Gryspeerdt et al., 2010

Monocytes/macrophages - Monocytes and macrophages engulf pathogens and apoptotic cells and produce immune effector molecules. Upon infection, monocytes are rapidly recruited to the infected site of the tissue, where they differentiate into tissue macrophages (Jakubzick et al., 2013; Yang et al., 2014). Monocytic cells have been shown to have a central role in the pathogenesis of several viruses such as human herpes virus 1 (HSV-1), Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV) (Linnavuori and Hovl, 1981; Savard et al., 2000; Bentz et al., 2006). Viral infection of monocytes and macrophages at different stages of differentiation has different outcomes and may result in the alteration of important cellular functions. Several studies showed that all equine PBMC subpopulations are susceptible to EHV-1 *in vitro,* but that cells of the monocytic lineage ($CD172a^+$cells), are the main infected subpopulation (Gryspeerdt et al., 2010; Vandekerckhove et al., 2010; van der Meulen et al., 2000; van der Meulen et al., 2006).

Dendritic cells - Dendritic cells (DCs) represent a heterogeneous population of immune cells which are specialized antigen-presenting cells (APCs) and most capable of efficiently activating naïve T cells to initiate immune responses (Rossi and Young, 2005; Mellman and Steinman, 2001). DCs are a target for many invading viruses, notably from the *Herpesviridae* family, such as HSV-1, HSV-2, VZV, EBV, HCMV, mouse cytomegalovirus (MCMV) and human herpesvirus 6 (HHV-6) (Rinaldo and Piazza, 2004). These viruses have developed several mechanisms to escape immune surveillance by DCs and to misuse these cells to disseminate through the body. In 1998, Steinbach and colleagues showed for the first time the infection of murine DCs with EHV-1 (Steinbach et al., 1998). Their data indicated that murine DCs may mediate a restricted infection. One year later, in 1999, Siedek et al. proved that EHV-1 is able to infect equine DCs in vitro (Siedek et al., 1999). Regarding the migratory life-style of DCs, the susceptibility to infection with EHV-1 possibly plays an important role in transporting infectious virus, from the mucosal surface of the respiratory tract to internal organs of the body including lymph nodes and endothelium of target tissues.

Lymphocytes - Lymphocytes play an essential role in combatting microbial infections. The interaction between viruses and lymphocytes is known to play a central role in on the one hand spread, and on the other hand control of viral infection within the host (Rudraraju et al., 2013; Shacklett et al., 2003). Previous studies proved that the main targets of infection, in the nasal

mucosa during an in vivo EHV-1 infection, are CD172a-positive mononuclear cells, which are either monocytes or dendritic cells, followed by T-lmphocytes and B-lymphocytes (Gryspeerdt et al., 2010). The study of Vandekerckhove et al., (2010) using the respiratory mucosa explants showed that monocytic cells ($CD172a^+$ cells) are the main targets followed by T-lymphocytes. Other studies on the replication in peripheral blood mononuclear cells have shown that EHV-1 replication is predominantly found in monocytes and B-lymphocytes after in vitro infection of PBMC (Yeo et al., 2013; Ma et al., 2010; Goodman et al., 2007). Van der Meulen and colleagues (2000) indicated that only 0.9 % of equine lymphocytes were infected with EHV-1 and less than 0.05 % produced infectious virus.

Latency

After an initial infection of respiratory epithelial cells, EHV-1 enters a latent state in sensory nerve-cell bodies within the trigeminal ganglia and in leukocytes (Allen et al., 2004). In latently infected horses, reactivation is followed by a shedding of the virus in nasal secretions and reoccurrence of viremia has been triggered by immune suppression upon administration of corticosteroids (Slater et al., 1994). Reactivation of a latent virus results in spreading of EHV-1 to susceptible animals which plays a crucial role in the epidemiology of this virus (Gibson et al., 1992; Edington et al., 1886). During latency, the expression of the EHV-1 genome is repressed and only a viral RNA transcribed from the immediate early (IE) gene, which is also named latency associated transcript (LAT), is present (Paillot et al., 2008). Detection of latent EHV-1 is possible by prolonged co-cultivation of permissive cells together with cells collected from blood, draining lymph nodes of the respiratory tract and trigeminal ganglia (Allen et al., 2004). The molecular mechanism by which EHV-1 enters into a latent relationship with its equine host cell is still not clear.

2.4. EHV-1 associated diseases

Respiratory disease

Infection of horses with EHV-1 results primarily in upper respiratory tract diseases such as rhinopharyngitis and tracheobronchitis (Allen et al., 2004). Young horses usually develop fever, serous to mucopurulent nasal discharge and swelling of draining lymph nodes, while older horses mainly show mild or subclinical disease (Coggins, 1979). In sporadically reported cases, EHV1 targets the pulmonary endothelium in young horses, which causes a severe pulmonary edema (Del Piero and Wilkins, 2001; Del Piero et al., 2000). Such severely affected horses may die in acute respiratory distress.

Abortion and neonatal syndrome

EHV-1 is considered as one of the most important infectious causes of abortion worldwide in horses (Leblanc, 1999). After cell-associated viremia, EHV-1 reaches endothelia in target organs, the uterus of a pregnant mare and the central nervous system. Adhesion molecules in endothelial cells of the pregnant uterus play an important role in the infection and are upregulated during an EHV-1 infection (Patel and Heldens, 2005). Widespread infection of endometrial blood vessels of the uterus leads to severe vasculitis and multifocal thrombosis, resulting in an abortion (Patel and Heldens, 2005). The period between infection and abortion differs from 9 days to 4 months but most mares abort within 21 days (Powell, 1991). During the last 4 months of pregnancy, there is a much higher chance (up until 95%) of abortion (Allen and Bryans, 1986).

Nervous system disorders

Several alphaherpesviruses, such as HSV-1, BoHV-1 and PRV are considered neurotropic viruses, being able to cause encephalitis by viral replication in neurons. However, EHV-1 does not cause myeloencephalitis by a specific neurotropism, but rather a marked endotheliotropism (Edington et al., 1986; Whitwell and Blunden, 1992; Wilson, 1997). Therefore, secondary replication of EHV1 in the endothelial cells of blood vessels of the nervous system is the first step in the development of nervous system disorders (Edington et al., 1986). All parts of the central nervous system may be affected by EHV-1.

3. Trafficking of leukocytes - Recirculation of leukocyte

EHV-1 is hijacking leukocytes in the respiratory tract in order to invade and to be transported via blood to internal target organs, such as the pregnant uterus and central nervous system. At present, it is not known how the virus is doing this. It could be that the leukocytes are just driven by a physiological process. However, it is also possible that the virus is taking over the migration of the virus for its own benefit. These important questions in the pathogenesis will be considered in the present PhD thesis. In this context, a good knowledge of cell trafficking is helpful in interpreting the results.

Cell trafficking is regulated by complex and heterogeneous mechanisms and is involved in a multitude of physiological as well as pathological processes, including embryogenesis, tumor metastasis, tissue formation, wound healing, and immune responses. In general, the migration of cells over substrata is a dynamic process that implies multiple steps (usually a five-step cycle) in which each "step" occurs simultaneously: (1) protrusion of the leading edge; (2) adhesion to the substrate; (3) contraction of the cytoplasm; (4) release from contact sites; and (5) recycling of membrane receptors from the trailing to the leading edge (Sheetz et al., 1999). Each step is regulated and mediated by one or more cyclical biochemical processes. Different cell types can adopt a variety of migration modes, which are commonly categorized on the basis of dynamics and the structure of the leading edge, and the underlying cytoskeletal organization (Friedl and Wolf, 2010; Weninger et al., 2014). Leukocytes are scattered throughout the body and have the potential to infiltrate any type of tissue. Their migration is integral to their function and is maintained throughout their life span. The mechanisms of leukocyte migration are essentially different from those of other cell types (Renkawitz and Sixt, 2010). Leukocytes use a migration mode (quick and frequent change in shape) that is classically termed "amoeboid" (Lämmermann and Sixt, 2009; Lämmermann et al., 2008). Indeed, this migration is induced by a variety of signaling mechanisms that receive and process information from the leukocyte environment and provides specific control of cytoskeletal and adhesion machineries within the cell (Petrie et al., 2009; Weninger et al., 2014). The adhesion of cells to the substratum is mediated largely by members of the selectin and integrin families (Ridley et al., 2003; Sheetz et al., 1998).

Some leukocytes (mainly monocytic cells) leave the blood circulation, differentiate into macrophages or DCs and patrol healthy tissues, including the mucosal epithelium of the respiratory tract (Imhof and Aurrand-Lions, 2004; Auffray et al., 2007; Yang et al., 2014; Randolph et al., 1999). A subset of monocytic cells (mainly DCs) capture antigens, and recirculate afterwards. They transport the antigens to the draining lymph nodes and blood circulation and present them to the immune system (Auffray et al., 2007; Jakubzick et al., 2013). Recirculation of monocytic cells is playing an important role in the pathogenesis of a generalized EHV-1 infection.

Leukocyte recirculation means that leukocytes leave the bloodstream and migrate through the tissues, where they fulfill their task as immune cells, and return to the bloodstream directly via capillaries or via efferent lymphatic vessels. They rapidly adapt their cell shape and migratory machinery to the different conditions of the microenvironment, which enables them to effectively traverse interstitial spaces at high speed. In general, the recruitment of leukocytes from the vasculature into tissues is fairly similar for the different leukocyte subpopulations (Chavakis et al., 2009). This extravasation process requires a complex cascade of adhesive events between the leukocytes and the endothelium including: (I) the initial selectin-dependent rolling and tethering of the leukocytes, (II) the chemokine-induced leukocyte activation, (III) the integrin-mediated firm adhesion and (IV) the transendothelial migration of leukocytes, which can take place in both a paracellular and a transcellular manner. Each of these steps (capture, rolling, slow rolling, firm adhesion and transmigration) appears to be necessary for effective leukocyte recruitment (Chavakis et al., 2009; Schmidt et al., 2013).

Leukocyte recruitment initiates with the capture of free flowing leukocytes and their subsequent rolling along the vessel wall. This is followed by firm leukocyte arrest, post-arrest modifications such as adhesion strengthening and intraluminal crawling, and finally transmigration into tissue. The initial attachment and rolling steps are initiated by interactions of endothelial E- and P-selectins and their counter receptors on leukocytes. The rolling step is reversible, unless followed by endothelial-presented chemoattractants and/or chemokines that activate leukocyte α4β1 (also called VLA4) and two members of the β2 integrin family, namely lymphocyte function-associated antigen 1 (LFA-1) and macrophage-1 antigen (Mac-1), to cause leukocyte arrest by binding to their cognate ligands, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), respectively (Leick et al., 2014). A detailed illustration of the perivascular extravasation in leukocyte migration is shown in Figure 6.

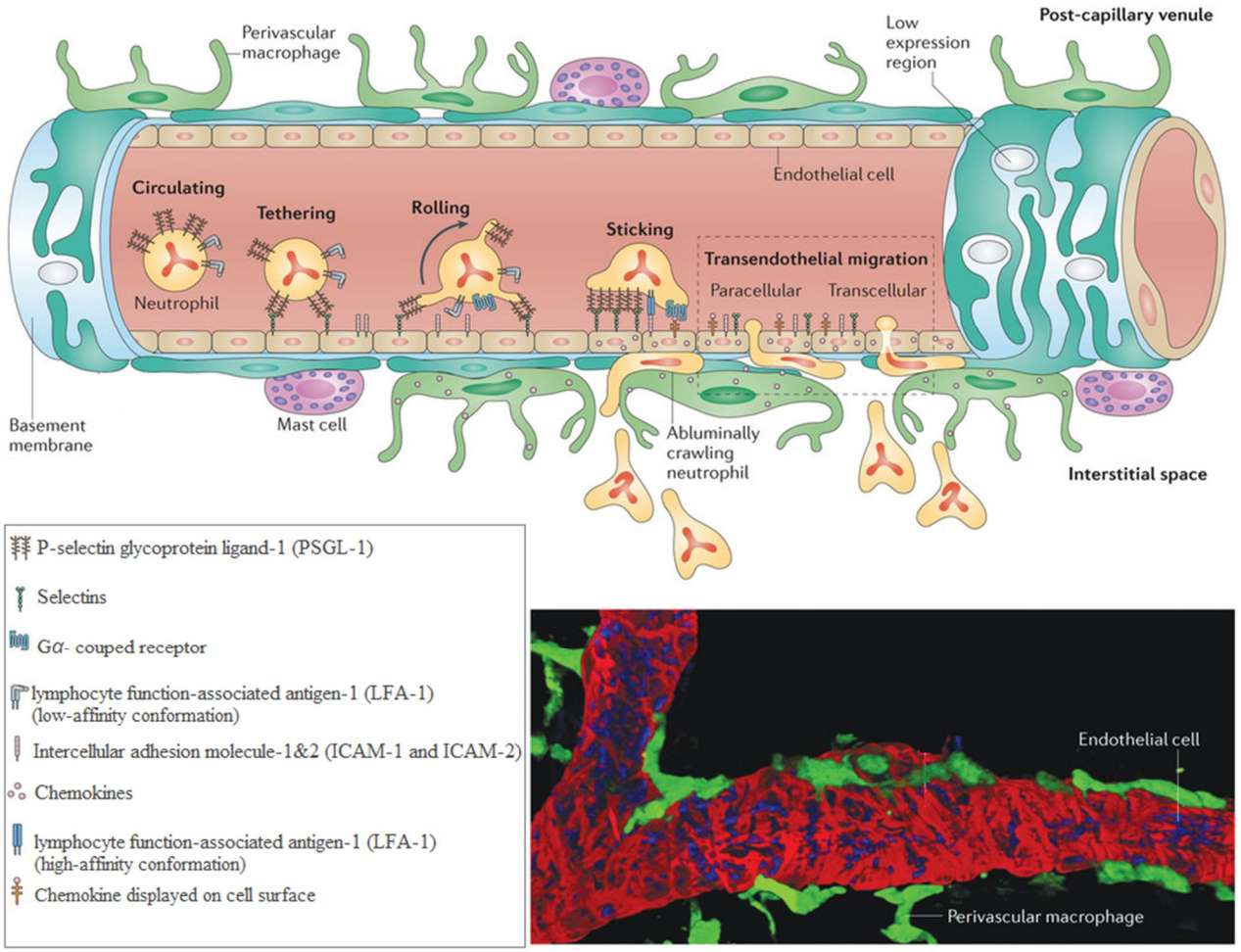

Figure 6. *Schematic representation of the perivascular extravasation unit, consisting of endothelial cells, the basement membrane, perivascular macrophages, and mast cells (above). The sequential steps in leukocyte emigration are controlled by interactions between specific molecules on leukocytes and their counter-receptors on endothelial cells. Following endothelial cell activation and the increased expression of P- and E-selectins, low-affinity adhesive interactions (capture and rolling) are elicited that subsequently lead to leukocyte activation, followed by firm adhesion and transendothelial migration. Confocal image of endothelial cells (blue) with perivascular macrophages (green) is shown underneath (Weninger et al., 2014).*

3.1. Leukocyte rolling and leukocyte activation during tethering

Leukocyte capture and rolling are mediated by selectins that belong to the C-type lectin family and interact with carbohydrate determinants on their ligands in a calcium-dependent manner. P-selectin is the primary adhesion molecule for capture and the initiation of leukocyte rolling (Sperandio, 2006; Schmidt et al., 2013). Inflammatory stimuli induce rapid exposure of P-selectin on the apical endothelial surface, which can support both capture and rolling in the absence of L-selectin (McEver, 2002). The most important ligand for selectins is P-selectin glycoprotein ligand-1 (PSGL-1), which is present as a homodimer on leukocytes and can bind to both P-selectin and E-selectin (Zarbock et al., 2007; Langer and Chavakis, 2009). Once leukocytes are captured, they may transiently adhere to the venular endothelium and begin to roll. In addition to promoting the initial interaction between activated endothelium and moving leukocytes, selectins might play a role in the induction of subsequent endothelial deformation, which would facilitate leukocyte arrest and transmigration towards peripheral tissues, and enhance the diffusion of soluble molecules between intravascular and peripheral compartments (Kaplanski et al., 1994). The three mentioned selectins share a similar structure and are named by the prefixes P (platelet), E (endothelial), and L (leukocyte), according to the cell type in which they were originally identified (Kelly et al., 2007). Several studies engaging antibody blockade of selectins demonstrated the participation of those in tethering and rolling of leukocytes.

3.2. Integrins in leukocyte migration

Integrins and their ligands

Integrins are a large family of heterodimeric transmembrane glycoproteins that attach cells to extracellular matrix (ECM) proteins or to ligands on other cells. Integrins comprise a large (120–170 kDa) α-subunit and a small (90–100 kDa) β-subunit. Mammalian genomes contain 18 α subunit and 8 β subunit genes, and 24 different α-β combinations have been identified at the protein level up until now. Although some subunits appear only in a single heterodimer, 12 integrins contain the β1 subunit, and 5 contain αV (Humphries et al., 2006; Luo et al., 2007; Plow et al., 2000; Hynes, 2002). The 24 integrin heterodimers are shown in Figure 7.

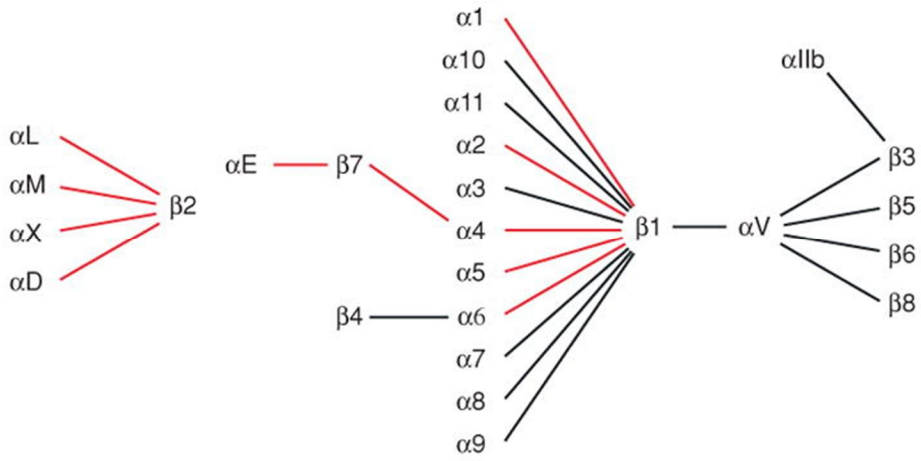

Figure 7. *The integrin receptor family. 18 α subunits combine with 8 β subunits to form 24 distinct heterodimers. Integrin heterodimers on immune cells are shown with red lines (Luo et al., 2007).*

Integrins are the important surface adhesion receptors mediating cell-matrix adhesion in metazoan; therefore their name denotes their relevance for maintaining the integrity of the cytoskeletal-extracellular matrix linkage (Berrier and Yamada, 2007). Integrins play a crucial role in a multitude of both physiological and pathological processes including cell migration, embryogenesis, development, tissue homeostasis, wound healing, leukocyte trafficking, immune responses, autoimmune diseases, cancer, and metastasis (Hynes, 2002; Hynes, 1992). Typical integrins include macrophage antigen-1, Mac-1 (αMβ2), leukocyte function–associated antigen 1 (LFA-1 or αLβ2), and very late antigen 4 (VLA-4 or α4β1). Mac-1 recognizes a more diverse set of ligands, including extracellular matrix proteins such as fibrinogen and fibronectin, as well as activated complement proteins such as iC3b. The ligands (or counter-receptors) for LFA-1 are the intercellular adhesion molecules (ICAMs) 1–5 that are expressed primarily on vascular endothelial cells. VLA-4 recognizes the vascular intercellular adhesion molecule (VCAM-1), whereas the major integrin on platelets, αIIbβ3, primarily binds fibrinogen (Abram and Lowell, 2009).

Integrin activation

The expression and activation of integrins are dynamically regulated, based on the activation of leukocytes. It has already been shown that integrins can exist in different ligand affinity states - low, intermediate and high (Luo et al., 2007). Crystal structures have revealed that integrin

heterodimers occur in an inactive, bent V-shape with the head close to the membrane-proximal regions of the legs, and are maintained by the α/β salt bridge at the inner membrane region and helix packing of the transmembrane region. The low affinity structure undergoes rapid, reversible conformational changes to increase ligand affinity, termed "activation" (Calderwood, 2004; Banno and Ginsberg, 2008). It is important to notice that leukocyte integrin activation usually takes place under blood flow conditions. Circulating leukocytes maintain their integrins in a low-affinity or in an inactive state to avoid nonspecific contact with uninflamed vascular walls. When they get to the inflammatory site, the chemokine-induced activation mediates a reversible change of the conformation of the integrins from the inactive, bent form to the extended form with intermediate affinity. This process prepares the integrin for binding to its endothelial ligand. The integrin undergoes a subsequent conformational alteration after binding to the ligand (outside-in signaling), culminating in the complete activation of the integrin (Barreiro and Sanchez-Madrid, 2009).

3.3. Role of ICAM-1 and VCAM-1 in leukocyte adhesion

The basis for the preferential binding of leukocytes to venular endothelial cells during inflammation, is related to the increased expression of some endothelial cell adhesion molecules in venules such as ICAM-1 and VCAM-1. Clustering of ICAM-1 and VCAM-1 on the endothelial cell has been observed as the leukocyte approaches the endothelial cell border (Muller, 2011; Muller, 2013; van Buul et al., 2010). ICAM-1 (CD54) is expressed on activated endothelial cells, high endothelial venules (HEV), T cells, B cells, monocytes/macrophages, granulocytes, and dendritic cells. ICAM-1–ligand interactions are involved in leukocyte firm adhesion, emigration through engagement with the endothelium, and also in leukocyte–leukocyte aggregation and activation (Pluskota and D'Souza, 2000; Kevil, 2003). ICAM-1 is a ligand for LFA-1 and is upregulated following inflammatory stimulation. Mac-1 also binds ICAM-1, however this integrin is a fairly promiscuous molecule capable of binding fibrinogen, fibronectin, and complement fragments (Diamond et al., 1993). VCAM-1 (CD106) is expressed on bone marrow stromal cells, myeloid progenitors, splenic dendritic cells, activated endothelial cells, as well as some lymphocytes. Upregulation of VCAM-1 occurs through de novo synthesis after cytokine stimulation (Beekhuizen and van Furth, 1993). Both VCAM-1 and ICAM-1 are involved in firm adhesion of leukocytes, to the apical surface of endothelial cells, through interactions with leukocyte integrins (Barreiro et al., 2002; Miller et al., 1995; Carman and Springer, 2004). Like

for ICAM-1, clustering of VCAM-1 induces intracellular signalling, leading to p38 phosphorylation, ROS production and protein tyrosine phosphatase 1B activation (van Furth 1993; Deem et al., 2007). It has already been indicated that the presence of the tetraspanins CD9 and CD151 is needed for optimal adhesive functions of ICAM-1 and VCAM-1 (Barreiro et al., 2005). Taken together, binding of leukocytes to either ICAM-1 or VCAM-1 induces multiple effects in the endothelium most of which serve to promote the interaction between the leukocyte and the endothelium as well as the subsequent transendothelial migration.

3.4. Leukocyte recruitment during inflammation

Inflammatory responses are the cornerstone of the body's defense mechanism against viral and bacterial pathogens as well as against physically, chemically and environmentally mediated tissue and organ damage. This crucial process can protect organisms by removing or neutralizing injurious agents. Recruitment of immune cells to sites of infection, immune reaction, or injury is complex and involves coordinated adhesive interactions between the leukocyte and the endothelial cell monolayer. It is well documented that the endothelium of a vessel wall is actively involved in supporting leukocyte transendothelial migration towards the site of inflammation (Springer, 1994; Leick et al., 2014). The site of leukocyte recruitment in most tissues is the postcapillary venule (Kluger, 2004). After binding of leukocytes to the endothelium, Ig-like cell adhesion molecules (CAMs) that are expressed on the apical surface of the endothelium, are clustered, recruited to sites of leukocyte adhesion, and concomitantly activated (Kluger, 2004). The main Ig-like CAMs on endothelium are inter-cellular adhesion molecule-1 (ICAM-1/CD54) and vascular cell adhesion molecule-1 (VCAM-1/CD106).

3.5. Leukocyte migration back to the blood circulation

Migration of leukocytes from the vascular lumen to the extravascular tissue is a vital component of the host's defence reaction to injury and infection. However, the migration of tissue-resident leukocytes (mainly APCs such as DCs) from mucosal tissues towards the blood circulation and lymph nodes have never been explored to date and the migration deserves more attention.

4. Mucosal immune cells

The mucosal immune cells of the equine respiratory mucosa are not characterized and defined up till now. In general, the mucosal surface of the respiratory tract is separated from the external environment by the epithelial barrier, which protects it using nonspecific defense mechanisms (mechanical cleaning, mucosal secretion, and others). The mucosa-associated lymphoid tissue (MALT) provide specific protective mechanism. Nasal-associated lymphoid tissue (NALT) is an organized lymphoid aggregated and infiltrated to the overlying epithelium in the nasal cavity. In humans, this tissue exists as a so-called diffuse NALT consisting of a collection of isolated subepithelial lymphoid follicles (Neutra, 1999; Debertin et al., 2003). The NALT has heterogeneous immunocompetent cells, including subepithelial B-lymphocytes, $CD4^+$and $CD8^+$T-lymphocytes, phagocytic APCs such as macrophages and diverse subsets of DCs. In addition, the overlying epithelium of mucosal follicles forms a specialized cell layer (i.e., follicle-associated epithelium) that has a loose structure which enables the contact between antigens and immune cells (Neutra, 1999; Bienenstock and McDermott, 2005).

The composition of quine nasal mucosal immune cells and and the susceptibility of these cells to an EHV-1 infection, and the effect of an EHV-1 infection on the behavior of these cells (migration and immunological response) have never been explored to date. By using the respiratory mucosa explants, it has been demonstrated that the majority of EHV-1-infected cells are monocytic cells which express surface marker CD172a (Vandekerckhove et al., 2010). CD172a belongs to the family of Signal Regulatory Proteins (SIRP) and is typically found on the surface of neurons and myeloid cells such as macrophages, DCs, monocytes and granulocytes (Barclay and Brown, 2006; Van Beek et al., 2005). Moreover, CD172a has also been described to be expressed on the cell surface of human, rat and equine mesenchymal stem cells (Vogel et al., 2003; Rooney et al., 2008; Claessen et al., 2015). The expression level of CD172a differs not only among monocyte and DC subtypes but also between different tissues (Seiffert et al., 2001; Epardaud et al., 2004; Bimczok et al., 2006; Lahoud et al., 2006; Saito et al., 2010). CD172a-positive macrophages are produced by differentiation of monocytes and their main function is phagocytosis of cellular debris and destroy pathogens. They are found between the epithelium and in the lamina propria of the respiratory mucosa (Holt, 1993). Several MHC class II and CD172a-positive DCs are situated

within the airway epithelium. DCs form a complex of APCs in the respiratory mucosa (Holt et al., 1990). DCs of mucosal surfaces are able to act as APCs after migration out of mucosal tissues to draining lymph nodes (Hamilton-Easton and Eichelberger, 1995). Upper respiratory mucosal DCs are greatly endocytic and one of the best at presenting antigenic peptides to naïve $CD4^+$ lymphocytes (von Garnier et al., 2005). The Langerhans cell (LC), probably the most expansively studied DC in the nasal mucosa is found in both the epithelial layer and in the lamina propria. Immature LCs are very effective in antigen binding and processing, but weak in stimulating resting T cells (Romani and Schuler, 1992). Howevere LCs lose their typical characteristics after maturation and change their structure, phenotype and functional capacities into those of DCs. The DCs function as ‘sensory cells’ of the immune system and identify danger signals. Besides their central role in the sensitization against environmental antigens, their ability to activate T cells in the airway mucosa allows them to sustain a prolonged inflammation (Huh et al., 2003). Due to their ability to take up antigens, their migratory activities and their ability to form close associations with T cells, DCs run the risk of becoming infected by and serving as transportation for viruses that infect mucosal surfaces such as human immunodeficiency virus (Pope et al., 1994).

5. Structure of the basement membrane and connection with epithelial cells

One of the crucial steps in the mucosal invasion mechanism of EHV-1 towards the underlying lamina propria is the crossing of the basement membrane barrier inside infected leukocytes. Until now the mechanism of the EHV-1 penetration process and the effect of the virus on different components of the basement membrane is unknown. In the current thesis, research was focused on the basement membrane barrier between epithelium and lamina propria and cell surface receptors of epithelial cells, integrins, which mediate cell-extracellular matrix adhesion. It is very well possible that an infection of EHV-1 in the epithelial cells are inducing changes in the integrin-anchors and the basement membrane in order to facilitate the passage of leukocytes in the infected aria. The basement membrane is a thin layer of a specialized extracellular matrix which encoded by a core set of approximately ten highly conserved genes. These genes encode predominantly large, insoluble secreted proteins which provide a scaffolding that shapes the basement membrane into sheet-like structures between 50 and 100 nm thick along cell surfaces. On electron micrographs, the basement membrane appears as electron-dense material in close proximity of the basal cell surface of epithelial cells. Conventional fixation results in a dense layer (lamina densa) separated from the plasma membrane by a translucent layer (lamina lucida), while a milder method of freeze substitution gives a more homogeneous appearance of the basement membrane (Hohenester and Yurchenco, 2013, Kelley et al., 2014). Therefore, by electron microscopy of glutaraldehyde-fixed and heavy-metal-impregnated thin sections, the basement membrane is typically seen as two thin structural layers. The first layer, the basal lamina (lamina basalis), is synthesized by epithelial cells and the second layer, the reticular lamina, is produced by fibroblasts. The basal lamina is subdivided into a clear lamina lucida directly under the epithelial cells and a structurally opaque lamina densa (Evans et al., 1990; Evans et al., 2010).

More than 50 proteins are known to make up the basement membrane of which the main components are: laminins, type IV collagen, nidogen and the heparan sulfate proteoglycans (HSPGs), and also often agrin, fibulins, fibronectin and other types of collagen (I, III, V, VI, VII, and XVIII) (Erickson and Couchman, 2000; Yurchenco et al., 2004; Yurchenco and Patton, 2009). The lamina lucida also contains extracellular proteins of cell adhesion molecules. The main cell

adhesion receptors which anchor cells to extracellular matrix and control a multitude of essential cellular functions by activating a variety of signaling pathways are integrins.

In general, the basement membrane separates epithelium, endothelium, peripheral nerve axons, fat cells and muscle cells from the surrounding stroma of any given tissue. Polarized epithelial cells, such as those in the respiratory tract, constantly remodel the basement membrane by secreting and proteolyzing its structural proteins. In turn, basement membrane proteins support epithelial cell-adhesion, survival and differentiation (Pozzi and Zent, 2011).

The main components of the basement membrane and the extracellular matrix of the lamina prporia are discussed below. A schematic overview of the basement membrane extracellular components is given in Figure 8.

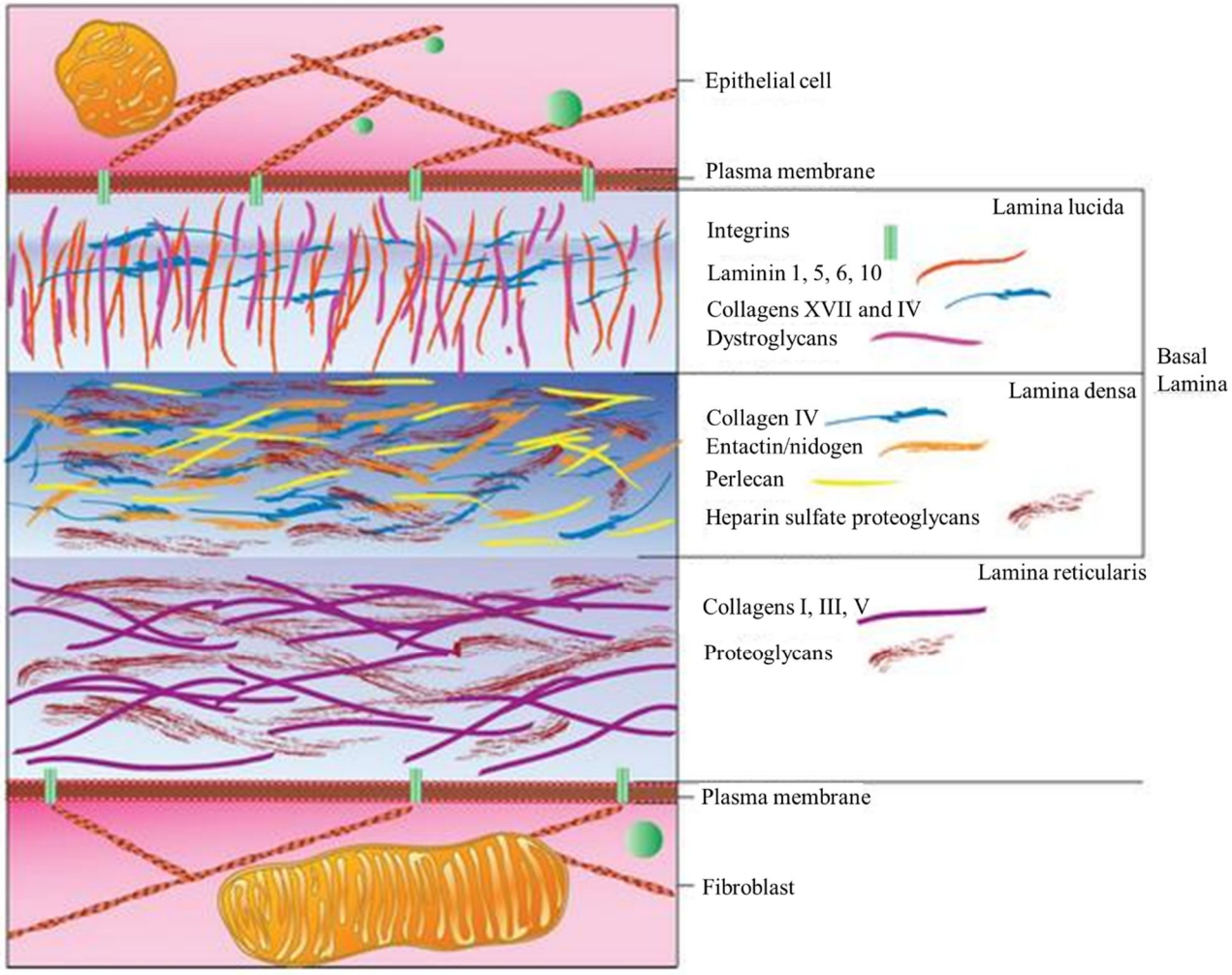

Figure 8. *Schematic representation of the basement membrane (Menter and DuBois, 2012).*

Laminins

Laminins are a family of large multidomain, heterotrimeric glycoproteins with molecular weights of 500–800 kDa. Sixteen trimeric isoforms have been described in mouse and human tissues, and these isoforms vary in their cell and tissue specificity. Each laminin isoform is composed of three chains, α, β, and γ, and each isoform exists in five, four, and three genetically distinct forms, respectively. Most vertebrates have five α, three γ and three to six β genes (Domogatskaya et al., 2012). Laminins stick to cells mainly due to the binding of the G domain of the α chains to integrins, dystroglycan, sulfated glycolipids or collagen type IV. Laminin molecules also interact with other components of the basal membrane matrix and underlying interstitial stroma, and as such contribute to the overall makeup. The N-terminal globular domains of the α1 and α2 chains as well as the globular domains VI (LN) of the α5 chains can adhere to many different integrin isoforms. Laminins contribute to the structure of the extracellular matrix and affect the adhesion, differentiation, migration, stability of phenotype, and resistance towards apoptosis of associated cells. (Halper and Kjaer, 2014). Due to the fact that a typical basement membrane has the same thickness as the dimension of a single laminin molecule, it is hypothesized that it is unlikely that laminins are standing erect on the cell surface (Hohenester and Yurchenco, 2013).

Collagens

Collagens are major proteins of the basement membrane and are certainly the most dominant. Years of research have discovered 28 different collagen types, and each type consists of homotrimers and heterotrimers that are formed by three polypeptide chains (known as α-chains). More than 40 distinct α-chains have been identified in humans as well as several other proteins containing domains similar to collagen (Ricard-Blum and Ruggiero, 2005; Mouw et al., 2014).
Collagens can be categorized into classes based upon their suprastructural organization which are shown in Table 3.

Table 3. General classification of collagen types (Mienaltowski and Birk, 2014).

Classification	Collagen types	Supramolecular structure
• Fibril-forming collagen	I, II, III	Striated fibrils
	V, XI	Striated fibrils, retain N-terminal regulatory domains
	XXIV, XXVI	Unknown
• FACIT collagens	IX, XII, XIV	Associated with fibrils, other interactions
• FACIT-like collagens	XVI, XIX, XXI, XXII	Interfacial regions, basement membrane zones
• Network-forming collagens		
- Basement membrane	IV	Chicken wire network with lateral association
- Beaded filament-forming	VI	Beaded filaments, networks
- Anchoring fibril	VII	Laterally associated anti-parallel dimers
• Hexagonal networks	VIII, X	Hexagonal lattices
• Transmembrane collagens	XIII, XVII, XXIII, XXV	Transmembrane and shed soluble ecto-domains
	Gliomedins, ectodysplasin	
• Multiplexin collagens (Endostatin–XV and -XVIII)	XV, XVIII	Basement membranes, cleaved C-terminal domains influence angiogenesis
• Other molecules with collagenous domains	XXVI, XXVIII	Collagenous domains in primarily non-collagenous molecules
	Acetylcholinesterase, adiponectin, C1q, collectins, surfactant protein, others	

FACIT: Fibril-associated collagen with interrupted triple helix.

Our research will focused on collagen type IV and the anchoring fibril (collagen type VII) of the basement membrane of the respiratory mucosa.

Collagen IV - Type IV collagen is the most often occurring element of the basement membrane. This typical collagen is also called 'network-forming collagen', due to its capacity to self-assembly into organized networks. Unlike most collagens, type IV collagen is only found in the basement membrane and forms supramolecular networks through a series of complex inter- and intramolecular interactions that influence cell adhesion, migration, and differentiation. Collagen VI interacts with many extracellular molecules including: collagens I, II, IV, XIV; microfibril-associated glycoprotein (MAGP-1); perlecan; decorin and biglycan; hyaluronan, heparin and fibronectin, as well as integrins and the cell-surface proteoglycan NG2. Based on the tissue-localization and large number of potential interactions, collagen VI has been proposed to integrate different components of the extracellular matrix, including cells (Ricard-Blum and Ruggiero, 2005; Mouw et al., 2014). Recent studies indicated that type IV collagen not only represents a structural protein providing tissue integrity but also affects the invasive behavior of trophoblast cells at the implantation site (Oefner et al., 2015).

Collagen VII - Type VII collagen forms an extended network of anchoring fibrils which consists of a central collagenous triple-helical domain flanked by two noncollagenous domains, NC1 and NC2. The NC1 domain contains multiple submodules with homology to known adhesive molecules including fibronectin type III-like repeats and the A domain of von Willebrand factor. NC1 subdomains also interact with other extracellular matrix proteins such as type I collagen and laminin-322. These anchoring fibrils are in close contact with hemidesmosome (Leineweber et al., 2011).

Proteoglycans

Proteoglycans are biological molecules composed of a specific core protein substituted with covalently linked glycosaminoglycan (GAG) chains (Schaefer and Schaefer, 2010). The primary biological function of proteoglycans derives from the biochemical and hydrodynamic characteristics of the GAG components of the molecules, which bind water to provide hydration and compressive resistance. In general, they classified into three major categories: (1) small leucine-rich proteoglycans, 2) modular proteoglycans, and 3) cell-surface proteoglycans. Being mostly extracellular, they are upstream of many signaling cascades and are capable of affecting intracellular phosphorylation events and modulating distinct pathways, including those driven by

bone morphogenetic protein/transforming growth factor superfamily members, receptor tyrosine kinases, the insulin-like growth factor-I receptor, and Toll-like receptors (Schaefer and Schaefer, 2010; Mouw et al., 2014). Proteoglycans are characterized by a core protein that is covalently linked to GAGs, which are long, negatively charged, linear chains of disaccharide repeats. Major GAGs include heparin sulphate, chondroitin sulphate, dermatan sulphate, hyaluronan and keratin sulphate. The main heparin sulphate proteoglycan is perlecan which its core protein has binding sites for type IV collagen, entactin/nidogen, and integrins (Mouw et al., 2014).

Entactin/nidogen

Entactin/nidogen accounts for 2 to 3% of the total amount of basement membrane protein. Nidogen-1 binds calcium ions and this calcium-binding activity of entactin may play a role in the matrix assembly process. Its major function appears to be the assembly of the basement membrane. The carboxyl globule binds tightly to one of the short arms of laminin at the inner rodlike segment. This same region is also believed to be responsible for the attachment of nidogen-1 to type IV collagen at approximately 80 nm from its carboxyl noncollagenous end. Nidogen-1 therefore could serve as a bridge between the two most abundant molecules in the basement membrane (Yurchenco and O'Rear, 1994; LeBleu et al., 2007).

Integrins

Integrins are a large family of heterodimeric transmembrane glycoproteins that act in a bidirectional fashion and are modulated by the mechanical properties of the cell-extracellular matrix interface (more details are discussed in section 3.2. Integrin in leukocyte migration). They are expressed on different cell types. Specialized epithelial cells express unique integrin ranges, however there are broad similarities in the pattern of integrin expression in most surface epithelia. At least seven different integrins are expressed on human airway epithelial cells (Sheppard, 2003). On the epithelia, their functions are: epithelial cell anchoring, fibroblast anchoring and leukocyte migration and homing. Signals from integrins also play essential roles in virtually every aspect of the behavior of epithelial cells, including survival, proliferation, maintenance of polarity, secretory differentiation, and malignant transformation (Hynes, 2002; Sheppard, 2003).

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CHAPTER II

AIMS OF THE STUDY

Equine herpesvirus type 1 (EHV-1) causes respiratory, nervous and reproductive problems in horses throughout the world, which lead to serious economic losses. Primary infection of EHV-1 occurs at the upper respiratory tract (URT) and is followed by a cell-associated viremia, which is the prerequisite for infection of endothelial cells of the pregnant uterus and central nervous system (CNS). Leukocytes play a crucial role in transporting the virus from the primary sites of replication to the internal target organs. It has been well documented that the majority of EHV-1-infected leukocytes are of the monocytic lineage. In addition, migration of these immune cells from the respiratory mucosa to the draining lymph nodes is important for the induction and initiation of both innate and adaptive immune responses.

The mucosal surface of the respiratory tract represents an important site of entry for a vast majority of pathogens and viruses in particular. Professional antigen presenting cells (APCs), such as dendritic cells (DCs) and monocytes/macrophages are a key factor in inducing primary immune responses against invasive pathogens. Most respiratory viruses restrict their replication to the mucosal epithelial cells, however some viruses such as EHV-1 are able to breach the basement membrane (BM) barrier by hijacking mononuclear immune cells.

The present study was designed to isolate and characterize mucosal monocytic cells in the respiratory mucosa (Chapter 3.1) and to compare their migration patterns with those of blood-derived monocytic cells and monocyte-derived DCs in nasal mucosal explants. Afterwards, the effect of EHV-1 infection on the migratory behavior of these three monocytic cell types in nasal mucosa was investigated (Chapter 3.2). In addition, the impact of an EHV1 infection on integrin alpha 6 and different components of the basement membrane (laminin, collagen IV and collagen VII) was examined in order to determine whether a mucosal EHV-1 infection may disrupt the barrier function of the underlying basement membrane (Chapter 4).

CHAPTER III

Monocytic cells in the nasal mucosa and effect of equine herpesvirus type 1 on their migratory behavior

A. Isolation and Characterization of Equine Nasal Mucosal CD172a$^+$ Cells

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Summary

The nasal mucosa surface is continuously confronted with a broad variety of environmental antigens, ranging from harmless agents to potentially harmful pathogens. This area is under rigorous control of professional antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages. Mucosal APCs play a crucial role in inducing primary immune responses and the establishment of an immunological memory.

In the present study, a detailed characterization of CD172a$^+$ cells, containing the APCs residing in the equine nasal mucosa was performed for the first time. CD172a$^+$ cells were isolated from collagenase-treated equine nasal mucosa fragments by MACS. Expression of surface markers was determined by flow cytometry and functional analysis was done by measuring the uptake of FITC conjugated ovalbumin (FITC-OVA). Cell surface phenotype of the isolated cells was as follows: 90% CD172a$^+$, 30% CD1c$^+$, 46% CD83$^+$, 42% CD206$^+$ and 28% MHC II$^+$. This clearly differs from the phenotype of blood-derived monocytes: 96% CD172a$^+$, 4% CD1c$^+$, 11% CD83$^+$, 9% CD206$^+$, 72% MHC II$^+$ and blood monocyte derived DCs: 99% CD172a$^+$, 13% CD1c$^+$, 30% CD83$^+$, 51% CD206$^+$ and 93% MHC II$^+$. The CD172a$^+$ nasal mucosal cells were functionally able to endocytose FITC-OVA but to a lesser degree than monocyte-derived DCs.

Together, these results demonstrate that the isolated CD172a$^+$ nasal mucosal cells resemble immature DCs in the nasal area.

1. Introduction

Dendritic cells (DCs), scattered throughout the peripheral tissues, act as sentinel cells and recognize a wide range of microorganisms. When pathogen invasion takes place, these still immature DCs (iDCs) can capture microorganisms via endocytic surveillance receptors, resulting in the classical intracellular lytic pathway that permits processing of antigenic peptides. Subsequently, the DC mature and migrate from tissue to the draining lymph node to present antigens to T-cells (Steinman, 1991). The DC subtype, immunological microenvironment and activation state determine the nature of the adaptive immune response produced, including a variety of effector responses, anergy or tolerance. The pivotal role of DCs in directing the immune response has generated great interest in DC biology and its relevance to many aspects of infection and immunity (Steinman and Banchereau, 2007).

It has already been shown that DCs are present at all mucosal surfaces throughout the body (Iwasaki, 2007). They are constantly exposed to both harmful and benign factors in contact with the mucosa (Długońska and Grzybowski, 2012; Soloff and Barratt-Boyes, 2010). Their role at these sites is to initiate and regulate innate immune responses, acquire foreign antigens, and present antigens in mucosa-associated lymphoid tissues (MALT) such as GALT (gut-associated lymphoid tissue), NALT (nasopharynx- or nose-associated lymphoid tissue), LALT (larynx-associated lymphoid tissue), BALT (bronchus-associated lymphoid tissue), and GENALT (genital-associated lymphoid tissue).

As the most potent antigen-presenting cell, DCs link the innate and adaptive immune responses after microbial infection. Mucosal DCs are specialized in inducing a local IgA response, which is extremely important in protecting the area exposed to the outer world. In horses, some pathogenic microorganisms such as alphaherpesviruses and arteriviruses are known to hijack mucosal monocytic cells and, by doing so, to invade the nasal mucosa (Vandekerckhove et al., 2011; Vairo et al., 2012). A clear understanding of the immunobiology of mucosal DC infection in the upper respiratory tract and how DCs are manipulated by viruses are therefore critical for designing effective prevention strategies for viruses that use the upper respiratory tract to enter the body not only in humans but also in domestic animals like the horse.

Cultivation and characterization of equine blood DCs in terms of morphology and function were first described in 1997, based on protocols used in humans (Siedek et al., 1997). Later on, in vitro differentiation of blood monocytes into DCs was accomplished using recombinant equine IL-4 and recombinant equine granulocyte macrophage colony stimulating factor (GM-CSF) (Mauel et al., 2006). The isolation of equine nasal mucosal DCs and their characterization have not been reported yet. Even nasal mucosal DCs in humans are still poorly understood (Allam et al., 2006; Faith et al., 2005).

In this report, we describe a novel technique to isolate nasal mucosal CD172a$^+$ cells from nasal mucosa. Phenotypical and functional characterization indicates that this cell population mainly consist of immature DC. This new technology will allow us to study the immunobiology of primary nasal mucosal APCs and to get a better insight in the intriguing interplay between viruses and this important cell population.

2. Materials and methods

2.1. Animals and nasal tissue collection

Nasal mucosae were collected from five horses at the slaughterhouse. Horses negative for nasal/ocular discharge and lung pathology were selected. All horses were between 4 and 7 years old, as determined by inspection of the dental incisive architecture (Muylle et al., 1996). For blood isolation, healthy horses were selected at the Faculty of Veterinary Medicine (Ghent University, Belgium). The experiments were authorized and supervised by the Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine of Ghent University (approval nr. 2013/007).

Collection of nasal mucosae was performed with minor modifications as described previously (Vandekerckhove et al., 2009). In brief, mucosal tissues from the deep intranasal part of the septum were collected. The tissues were transported in phosphate-buffered saline (PBS), supplemented with 1 µg/ml gentamicin (Gibco), 1 mg/ml streptomycin (Certa), 1000 U/ml penicillin (Continental Pharma), 5 µg/ml fungizone (Bristol-Myers Squibb), and 10% fetal calf serum to the laboratory. To avoid spontaneous migration of DCs, the tissues were kept on ice during transport. Nasal tissues were cut into strips of about 2 × 6 cm. For removing residual blood, the tissue fragments were

thoroughly rinsed with subsequently CMF-PBS (calcium magnesium free phosphate buffered saline) and RPMI-1640.

2.2. Preparation of single cell suspension from nasal tissues

The nasal tissues were chopped into 3mm^2 and then transferred to a bottle containing CMF-PBS, 5% FCS, 1 µg/ml gentamycin, 1 mg/ml streptomycin, 1000 U/ml penicillin and 10mM EDTA (VWR BDH Prolabo) and incubated at 37°C for 30 min while shaking at 250 rpm (Unimax 2010, Heidolph Brinkmann, Germany). Tissues were then transferred to new beakers containing CMF-PBS, 5% FCS, 1 µg/ml gentamycin (Gibco), 0.1 mg/ml streptomycin (Certa) and 100 U/ml penicillin (Continental Pharma), 30µg/ml DNase I (Sigma-Aldrich), collagenase type IV (220U/ml, Gibco, USA) and incubated for 2 hours at 37°C on a shaker platform (Unimax 2010, Heidolph Brinkmann, Germany). The supernatant from digests was centrifuged at 4°C for 10min at 300×g, and the cell pellet was re-suspended in PBS. A cell strainer with a pore size of 40µm (BD Biosciences, San Jose, CA) was used for obtaining a uniform single cell suspension. The suspension was layered over Ficoll-Paque™ PLUS gradient (density 1.077; GE Healthcare, Life Sciences) and the interface cell layer was then collected and washed three times with CMF-PBS. The single cell suspension was kept on ice until MACS separation and flow cytometery analysis.

2.3. Purification of nasal mucosal CD172a$^+$ cells by MACS

After digestion, single cell populations were stained with anti-CD172a mAb (Tumas et al., 1994) and then positive cells were selected with anti-mouse IgG beads. (Miltenyi Biotech Ltd). For MACS preparation, first mouse monoclonal anti-CD172a (VMRD, clone DH59B, 1:100, IgG1) was added to the isolated nasal mucosal cells and after incubation during 20 min at 4°C, goat anti-mouse IgG magnetic beads were added according to the manufacturer's instructions. After 20 min incubation at 4°C, cells were washed and passed over a MACS column. Positively selected cells were isolated and suspended in RPMI-1640 containing 10% fetal calf serum (FCS), 1 µg/ml gentamycin, 0.1 mg/ml streptomycin and 100 U/ml penicillin and then cultured in 6 well plates for 6 days.

2.4. PBMC isolation and DC generation

As a control population, equine blood-derived monocytes and monocyte derived DCs were prepared with minor modifications as previously described (Dietze et al., 2008). Briefly, monocytes were isolated from 50 ml heparinized-blood (100µl heparin). Blood was diluted 1 in 1 with cold CMF-PBS and layered onto Ficoll-Paque™ PLUS gradient (density 1.077; GE Healthcare, Life Sciences), then centrifuged at RT for 30 min at 2100 rpm. The PBMC interface was washed twice in phosphate-buffered saline and resuspended in RPMI-1640 containing 10% fetal calf serum (FCS), 1 µg/ml gentamycin, 0.1 mg/ml streptomycin, 100 U/ml penicillin. Cells were cultured at 37°C in 5% CO2 for 1-2 hours. Non-adherent cells were removed from the plate and cultured in new dishes for subsequent adherence of monocytes. The adherent monocytes were kept in RPMI-1640 with 10% FCS, 1 µg/ml gentamycin, 0.1 mg/ml streptomycin, 100 U/ml penicillin for 24 hours. Monocyte derived DCs were obtained by adding recombinant equine GM-CSF (Kingfisher Biotech, USA) and IL-4 (R&D Systems, UK) at concentrations of 20 ng/ml and 10 ng/ml, respectively. After 2 days, the medium was replaced by new cytokine-containing medium. All cells were collected by accutase detachment solution (Sigma-Aldrich), counted and analysed for their viability by flow cytometry using propidium iodide.

2.5. Immunophenotyping by flow cytometry

Immunophenotyping of equine monocyte derived DCs and equine nasal mucosal CD172a$^+$ cells were performed by membrane immunofluorescence staining of cell markers. 1-5×10^5 cells were used per marker. The following primary antibodies (Abs) were enclosed: mouse monoclonal anti-human CD1c (Biolegend, clone L161, 1:50, IgG1), mouse monoclonal anti-human CD83 (Biolegend, clone HB15e, 1:50, IgG1), mouse monoclonal anti-CD172a (VMRD, clone DH59B, 1:100, IgG1), mouse monoclonal anti-human CD206 (Biolegend, clone 15.2, 1:50, IgG1), and mouse monoclonal anti-equine MHC Class II (Novus Biological, clone CVS20, 1:100, IgG1) and an appropriate isotype-matched control (IgG1) mouse monoclonal anti-PRV gD antibody 13D12 (Nauwynck and Pensaert., 1995). FITC-labelled goat anti-mouse IgG1 (Immunotech) were used as secondary antibody. At least 10,000 cells were analysed using a FACS Canto flow cytometer (Becton Dickinson Immunocytometry systems). The data were further analysed with the FACS Diva software.

2.6. Antigen uptake

There is a variety of endocytic routes that can be used by DCs to take up antigens (Werling et al., 1999; Michalek et al., 1991). To examine the endocytic abilities of the different nasal cell populations in this study, FITC conjugated ovalbumin (FITC-OVA; Molecular Probes, Eugene) was used. The uptake of this tracer molecule was examined using the methodology described by Werling et al. (1999). Cells were harvested from equine nasal mucosa as mentioned above. After incubation of 1×10^5cell/ml with FITC-OVA (1 mg/ml) for 1 hour at 37°C, uptake was quantified as mean fluorescence intensity (MFI). Background FITC signal was assessed by incubating cells in FITC-OVA at 4°C. This value was subtracted from the value measured at 37°C. All incubations were performed in PBS-5% FCS. 10,000 events were acquired for each sample using a flow cytometer (Becton Dickinson Immunocytometery systems). To verify that the flow cytometry-based FITC signal represented internalized OVA, cells were also analysed by confocal fluorescence microscopy.

2.7. Statistical analysis

We designed an experiment to compare FITC-OVA uptake in nasal mucosal cells and peripheral blood mononuclear cells, and to analyse the response of these cells and subpopulations after treatment with IL-4/GM-CSF and LPS. To this end, an experiment studying three factors (cell type, subpopulations and stimulators) in completely randomized design (CRD) with four replicates was carried out. Duncan's multiple range tests were used to compare the means. Freshly isolated and two days cultured nasal mucosal cells were compared using an unpaired two-tailed Student’s t-test. P values equal or lower than 0.05 were considered to be statistically significant. All statistical analyses were carried out using SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Isolation of nasal mucosal CD172a$^+$ cells by magnetic-activated cell sorting

CD172a$^+$ cells were isolated from equine nasal mucosal cell suspensions by magnetic cell sorting after CD172a labeling. To obtain a sufficient purity of enriched cells, the concentration of CD172a antibodies (VMRD, clone DH59B, 1:100, IgG1), anti-mouse IgG microbeads (20µl per 10^7cells) and the type of separation column (LS, MidiMACS) were optimized. The number of cells that were obtained after collagenase treatment (220U/ml) of two strips (2 × 6 cm) in 20ml was approximately 7×10^7cells/ml. MACS resulted in 2×10^7 CD172a$^+$cells and 5×10^7 CD172a$^-$ cells. This methodology led to a purity of > 90% CD172a$^+$ positive cells and a viability of > 85 %.

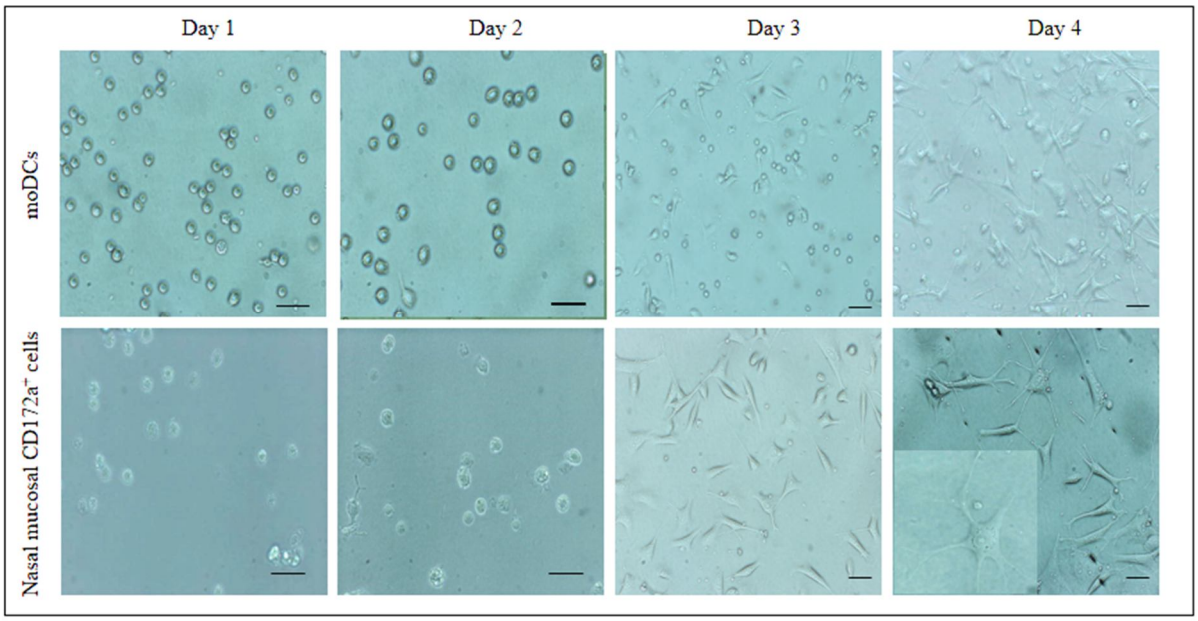

Figure 1. *Monocyte-derived dendritic cells and equine nasal mucosal CD172a$^+$ cells. Peripheral blood mononuclear cells were incubated for 2 hours at 37 °C. The non-adherent cells were washed away and the adherent cells were cultured for five days in the presence of rEq GM-CSF (20 ng/ml) and rEq IL-4 (10 ng/ml). Equine nasal mucosal CD172a$^+$ cells were isolated from nasal mucosa by digestion and magnetic activated cell sorting and cultured for five days without cytokines. Scale bar: 20 µm.*

3.2 Morphology of equine nasal mucosal CD172a$^+$ cells

The morphology of the equine nasal mucosal CD172a$^+$ cells was examined by means of light microscopy during a period of five days (Figure 1). Light microscopy was used to calculate cell numbers and to determine viability (trypan-blue staining of dead cells). Cultures showed large elongated cells extending their dendrites onto the plastic after 24 hours. After 72 hours, the cells became more round to triangular and almost always contained vacuoles and prominent pseudopodia. Although cells varied in size and shape, each cell had numerous dendrites which were spread in all directions. Interestingly, most of the CD172a$^+$ cells were moving slowly during the first 2 days of culture. Afterwards, they were crawling more actively using dendrites and pseudopodia up till 3-4 days (Videos 1 and 2). After 5 days, most of them were attached to the culture dish.

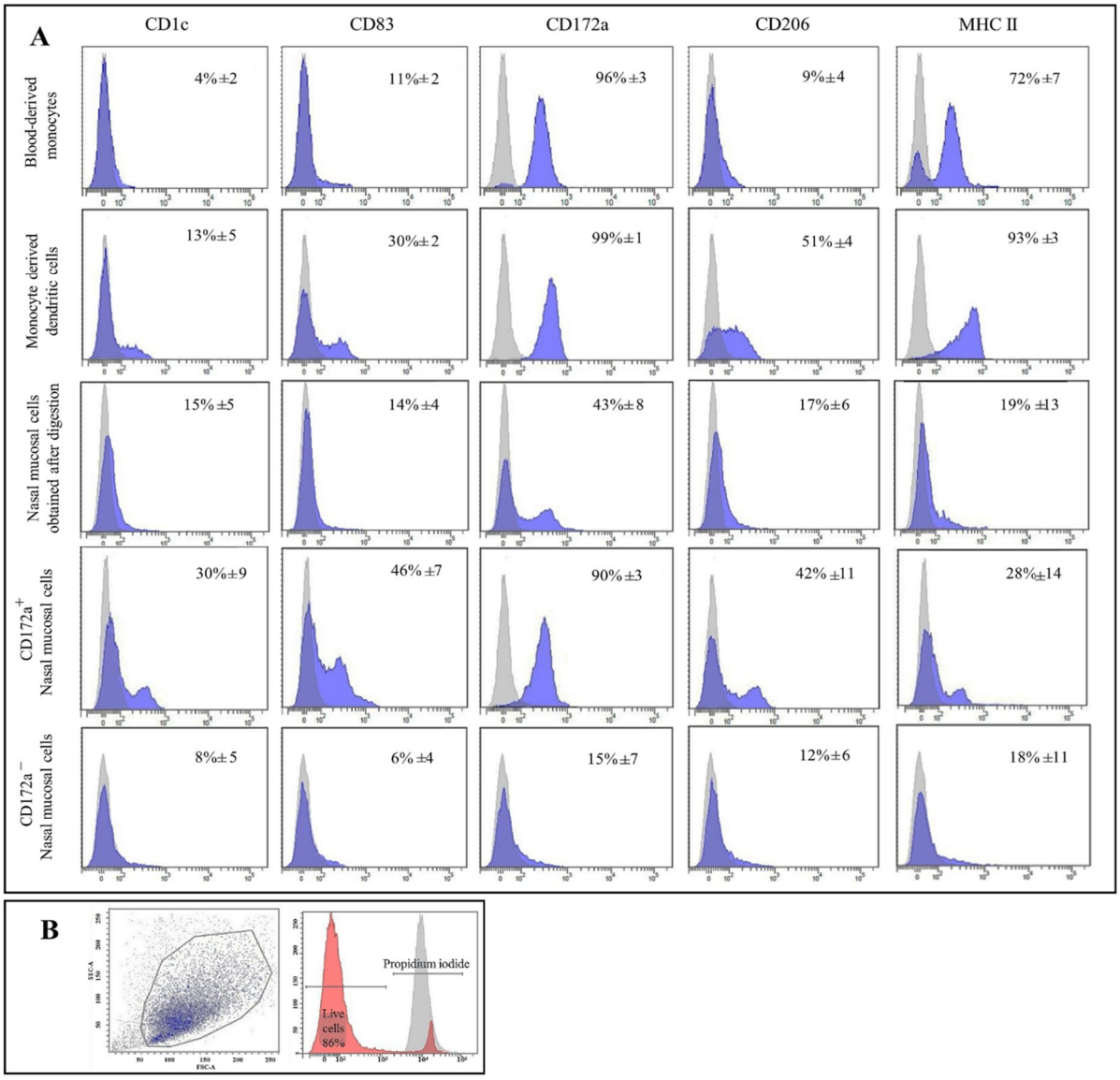

Figure 2. *Flow cytometric analysis of equine blood-derived monocytes, monocyte derived dendritic cells, equine nasal mucosal cells and CD172a^{+} positive and negative subpopulation (A). Forward and side scatter characteristics of nasal mucosal cells and histogram of propidum iodide which is excluded as dead cells (B). Light grey filled histograms illustrate isotype-matched controls. The data shown are representative from five different horses.*

3.3. Phenotyping of equine nasal mucosal CD172a^{+} cells

Antibodies against the DC markers CD1c, CD83, CD172a, CD206 and MHC II were used to characterize equine nasal mucosal CD172a^{+} cells (Figure 2). The expression of CD172a has been used to identify DC subsets in many mammalian species (Milling et al., 2010). Isolation of nasal

mucosal cells by magnetic-activated cell sorting with anti-CD172a antibodies resulted in a purity that was higher than 90%. The markers CD1c, CD83, CD206 and MHC II were expressed on 30%, 46%, 42% and 28% of nasal mucosal cells, respectively. As a point of comparison, equine fresh blood-derived monocytes and blood monocyte derived DCs were included. The phenotype of blood-derived monocytes was 96% CD172a$^+$, 4% CD1c$^+$, 11% CD83$^+$, 9% CD206$^+$, 72% MHC II$^+$ and of monocyte derived DCs was 99% CD172a$^+$, 13% CD1c$^+$, 30% CD83$^+$, 51% CD206$^+$ and 93% MHC II$^+$. Both blood-derived populations were clearly different from the isolated nasal mucosal CD172a$^+$ cells. The expression of CD1c and CD83 were considerably higher in CD172a$^+$ cells compared with blood-derived monocytes and blood monocyte derived DCs whereas CD206 was expressed at the same level in CD172a$^+$ cells and monocyte derived DCs. Furthermore, the MHC II expression was lower in CD172a$^+$ cells than in both blood-derived monocytes and blood monocyte derived DCs.

3.4. Functional characterization

The ability of immature DCs to take up antigens in the nasal mucosa is crucial for generating an immune response to invading pathogens. Several pathways exist for antigen uptake. FITC-OVA is generally used as a measure for receptor-mediated endocytosis. The FITC-OVA model antigen is preferentially internalized by macropinocytosis (Lutz et al., 1997), which has been described as the most efficient way of soluble antigen internalization (Mc Ever, 1992). To evaluate endocytic activity of nasal mucosal cells, either freshly isolated or after cultivation, the uptake of FITC-OVA was measured in newly isolated cells and after culturing two days (Figure 3). There was no significant difference between fresh and two days old nasal mucosal cells. In CD172a$^+$ cells, the uptake was three times higher than in CD172a$^-$ cells. To compare the isolated nasal mucosal cells with another well-characterized population of leukocytes containing DCs, equine blood mononuclear cells were enclosed. Both populations were treated for two days with eqIL-4 (10ng/ml) / eqGM-CSF (20ng/ml), two well-known DC differentiation factors, and/or 12 hours with LPS (1µg/ml), a well-known DC activation factor (Figure 4). The ability to take up FITC-OVA increased in all cells when treated with eqIL-4 / eqGM-CSF. This increase was significant in CD172a$^+$ positive cells. Cells activated with LPS decreased their ability of antigen uptake, especially in nasal mucosa CD172a$^+$ cells.

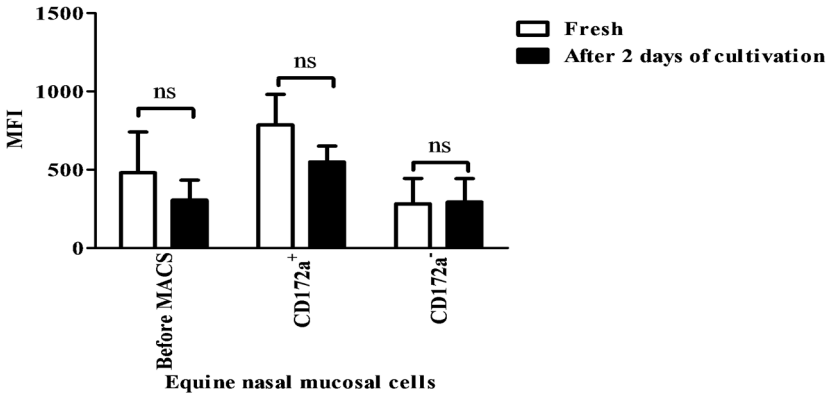

Figure 3. *Effect of aging on uptake of FITC ovalbumin (FITC-OVA) in isolated equine nasal mucosal cells. Nasal mucosal cells were adjusted to a concentration of 1×10^5 cells per ml and incubated with FITC-OVA (1mg/ml) for 1 hour at 37°C or 4°C. Mean fluorescence intensity (MFI) = MFI (37°C) - MFI (4°C). Statistical analysis for comparison between freshly isolated cells and after culturing two days was performed using unpaired two-tailed Student's t-test. The data shown are representative for three different experiments. ns: non significant.*

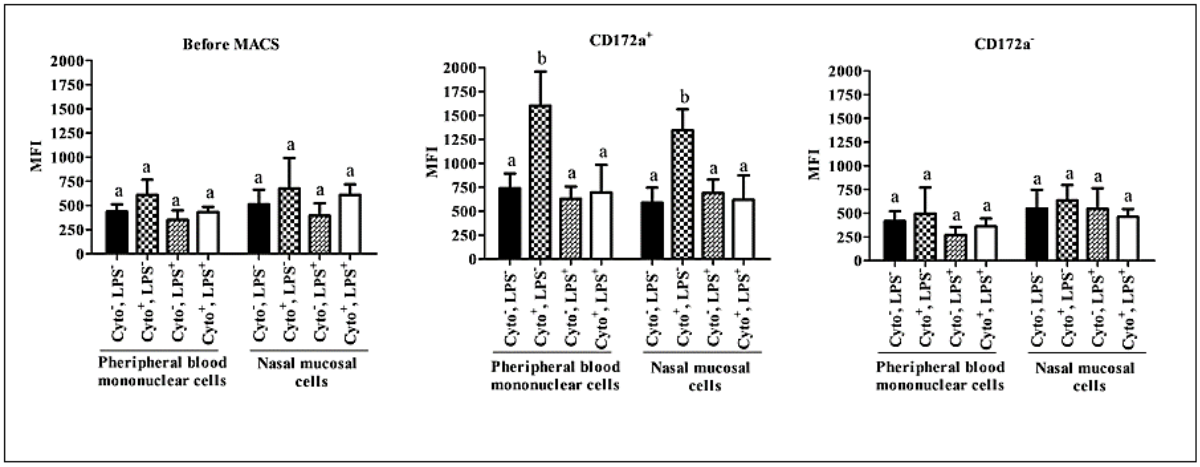

Figure 4. *Comparison of FITC-OVA uptake in three subpopulations of equine peripheral blood mononuclear cells and equine nasal mucosal cells. All cells were cultivated with or without eq IL-4 (10ng/ml) & eq GM-CSF (20ng/ml) for two days and stimulated with or without LPS (1µg/ml) for 12 hours. Then, adjusted to a concentration of 1×10^5 cells per ml and incubated with FITC-OVA for 1h at 37°C or 4°C. Mean fluorescence intensity (MFI) = MFI (37°C) - MFI (4°C). Error bars represent standard deviations (SD). Values above columns followed by the same letter are not significantly different ($P > 0.05$) using the Duncan test. The data shown are representative for four different experiments.*

4. Discussion

The nose of a horse is the entry site for a large variety of airborne antigens, including a range of infectious microorganisms, especially viruses such as equine herpesvirus 1 (EHV-1) and equine arteritis virus (EAV) (Gryspeerdt et al., 2010; Vandekerckhove et al., 2011; Vairo et al., 2012). Both EHV-1 and EAV have a tropism for nasal mucosal leukocytes (CD172a$^+$ cells and T-lymphocytes) and misuse these cells for viral transport through the basement membrane (BM) barrier (Vairo et al., 2013; Gryspeerdt et al., 2010). Within this mucosal environment, DC play a pivotal role by initiation and modulation of immune responses via sampling and presenting peptides in an immunogenic form to the adaptive immune system.

In large animals, the nasal mucosal immune cells have not been well-characterized. In the present study, a new methodology consisting of a combination of collagenase digestion and MACS was developed to isolate equine nasal mucosal CD172a$^+$ cells and to characterize them by morphological analysis, phenotyping and functional testing.

CD172a is a transmembrane regulatory protein expressed primarily by myeloid cells such as macrophages, DCs, monocytes and granulocytes (Barclay and Brown, 2006; Van Beek et al., 2005). The expression level of CD172a differs not only among DC subtypes but also between different tissues (Seiffert et al., 2001; Epardaud et al., 2004; Bimczok et al., 2005; Bimczok et al., 2006; Lahoud et al., 2006; Saito et al., 2010). In pigs, lamina propria DCs in the small intestine are mainly CD11b$^+$/CD172a$^+$ whereas DCs present in the trachea mucosa are dominated by CD16$^+$/CD11b$^-$/CD172a$^-$ populations (Bimczok et al., 2005; Bimczok et al., 2006). It has been also reported that CD172a$^+$ DCs are the primary immunogenic DCs that migrate out of tissues to promote Th2 responses in draining lymph nodes (Raymond et al., 2009). In the present study, we examined the morphology, phenotype and function of horse CD172a$^+$ cells. As comparison, we used blood derived DCs generated from peripheral blood monocyte cells using IL-4 and GM-CSF. In the past, the morphology of equine monocyte-derived DCs was shown to be veiled cells with pseudopodia (Mauel et al., 2006). Enzymatic digestion and CD172a magnetic activated cell sorting of equine nasal mucosal cells resulted in a population of cells that exhibited an immature phenotype during the first two days of activation but a clear morphological appearance of DCs after four days. Temporary cytoplasmic extrusions and various branched protoplasmic extensions were observed in the isolated cells, which are typical for DCs.

Equine DC and macrophage differentiation markers have been reported and characterized previously (Siedek et al., 1997; Mauel et al., 2006; Lunn et al., 1998; Kydd et al., 1994; Ibrahim and Steinbach, 2007). Based on the expression of different markers, one of the important differences between equine blood monocyte derived DCs and equine nasal mucosal monocytic cells was CD1c which is originally classified as a Langerhans cell marker in the epidermis but was later on also detected on human nasal mucosal DCs (Faith et al., 2005; Jahnsen et al., 2004; Peiser et al., 2003). DCs positive for CD1c are located in both the epithelium and lamina propria of the human respiratory tract (Jahnsen et al., 2004). In the present study, the percentage of CD1c$^+$ cells was significantly higher in isolated CD172a$^+$ cells from nasal mucosae than in blood monocyte derived DCs. The comparable high expression of mannose receptor CD206 in both monocyte derived DCs and our isolated nasal mucosal CD172a$^+$ cells illustrated their rather immature nature. CD83 is one of the well-known maturation markers on human and murine DCs and is markedly up-regulated together with co-stimulatory molecules CD80 and CD86 during DC maturation (Zhou and Tedder, 1995; Berchtold et al., 1999). Saalmüller and colleagues have demonstrated that antibodies against human CD83 and other markers could be applied to equine DCs (Saalmüller et al., 2005). The expression of CD83 in equine nasal mucosa CD172$^+$ cells was somewhat higher than immature monocyte derived DCs. In the present investigation, the expression of MHC class II on isolated nasal mucosal CD172a$^+$ cells was low. By a double immunofluorescence staining for CD172a and MHC II markers on fixed cryosections of nasal mucosa of healthy slaughterhouse horses, we confirmed the general lack of MHC II on CD172a$^+$ cells in situ (data not shown). Only a small subset of CD172a$^+$ cells within the nasal mucosae expressed MHC II with the majority having a low level expression. These findings agree with previous observations done in human respiratory mucosa cells (Faith et al., 2005).

Immature DCs are known to take up antigens via receptor-mediated endocytosis and macropinocytosis. Upper airway mucosal DCs are more endocytic than their lung counterparts and one of the best at presenting antigenic peptides to naïve CD4$^+$ lymphocytes (von Garnier et al., 2005). DC maturation leads to the down-regulation of the antigen-uptake machinery, up-regulation of adhesion and costimulatory molecules, maturation marker CD83 and peptide-MHC complexes as well as the polarization of different T-cell subsets (Banchereau and Steinman, 1998; Berchtold et al., 1999). In the present study, we measured endocytic activity of freshly isolated nasal mucosal CD172a$^+$ cells and after two days cultivation. Our analysis revealed that nasal mucosal CD172a$^+$

cells took up three times more antigens than CD172a$^-$ cells and culturing did not influence the uptake activity. Such high uptake could be due to the immature nature of CD172a$^+$ cells in the nasal area.

It is well documented that professional APCs, notably immature DCs in peripheral tissues, are highly endocytic and internalize a wide variety of antigens, whereas mature cells have downregulated this activity (Banchereau and Steinman, 1998; Burgdorf et al., 2007; Blum et al., 2013). DC generated with IL-4/GM-CSF express high levels of MHC class I and II and increase the capacity of antigen uptake by macropinocytosis and receptor-mediated endocytosis (Basak et al., 2002). In contrast, LPS stimulation decreases the endocytosis activity and activates migration of DCs (Anderson et al., 2009; Kamphorst et al., 2010). To identify the effect of differentiation and activation for efficient antigen capture and functionality of the cells, we studied the uptake capacity of isolated nasal mucosal cells and compared them with peripheral blood mononuclear cells. Both populations were treated with IL-4 / GM-CSF and stimulated with LPS. FITC-OVA was used as a measure for endocytosis activity of all cell populations. Our results revealed that the ability of antigen uptake increased in CD172a$^+$ cells when treated with IL-4 / GM-CSF and decreased upon LPS activation. In CD172a$^+$ cells, FITC-OVA uptake was significantly greater than CD172a$^-$ cells. Therefore, it can be concluded that IL-4 / GM-CSF activated uptake of FITC-OVA. In contrast, LPS is turning down the antigen uptake in cytokine-treated cells. These findings are clearly in agreement with what has been described in human or mice (Ahn and Agrawal, 2005; Lutz et al., 1996). Furthermore, we showed that in cytokine differentiated and/or LPS stimulated blood monocyte derived CD172a$^+$ cells antigen uptake was always higher than in nasal mucosal CD172a$^+$ cells. It looks like nasal mucosal CD172a$^+$ cells under steady-state conditions sample much antigens and internalize them without directly expressing them via MHC II (low expression level in the present study). These cells resemble immature DCs that are professional antigen captures and processors at equine nasal mucosal surfaces and carry their antigens to draining lymph nodes in order to initiate innate immune response. However, the precise function of these cells in vivo remains to be defined.

In conclusion, magnetic-activated cell sorting of equine nasal mucosal CD172a$^+$ cells ensures the isolation of viable and functionally intact cells that retain their semi-immature state. These cells are thus ideal for elucidating the functional and phenotypic properties of mucosal APCs, not only

in equine but also in other animals and humans. They offer a unique study ground to elucidate the cellular and molecular mechanisms responsible for their differentiation, immunostimulatory capabilities, and the migration of these crucially important immune conductors. Our observations have significant implications for a better understanding of the initiation of nasal mucosal immune responses. Furthermore, the homogeneous and well-defined population of nasal mucosal CD172a$^+$ cells obtained with our protocol will be suitable to examine how viruses may hijack these cells during invasion and immune evasion.

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Appendix A. Supplementary data

Supplementary data (Videos) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2013.12.001.

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B. Impact of equine herpesvirus type 1 (EHV-1) infection on the migration of monocytic cells through equine nasal mucosa

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Summary

The mucosal surfaces are important sites of entry for a majority of pathogens, and viruses in particular. The migration of antigen presenting cells (APCs) from the apical side of the mucosal epithelium to the lymph node is a key event in the development of mucosal immunity during viral infections. However, the mechanism by which viruses utilize the transmigration of these cells to invade the mucosa is largely unexplored.

Here, we establish an *ex vivo* explant model of monocytic cell transmigration across the nasal mucosal epithelium and lamina propria. Equine nasal mucosal $CD172a^+$ cells ($nmCD172a^+$ cells), blood-derived monocytes and monocyte-derived DCs (moDCs) were labeled with a fluorescent dye and transferred to the apical part of a polarized mucosal explant. Confocal imaging was used to monitor the migration patterns of monocytic cells and the effect of equine herpesvirus type 1 (EHV-1) on their transmigration. We observed that 16-26% of mock-inoculated $nmCD172a^+$ cells and moDCs moved into the nasal epithelia, and 1-7% moved further in the lamina propria. The migration of EHV-1 inoculated monocytic cells was not increased in these tissues compared to the mock-inoculated monocytic cells. Immediate early protein positive (IEP^+) cells were observed beneath the basement membrane (BM) 48 hours post addition (hpa) of moDCs and $nmCD172a^+$ cells, but not blood-derived monocytes. Together, our finding demonstrate that monocytic cells may become infected with EHV-1 in the respiratory mucosa and transport the virus from the apical side of the epithelium to the lamina propria en route to the lymph and blood circulation.

1. Introduction

Nasal mucosal tissue is heavily populated with immune cells, which are continuously bombarded with a flow of foreign antigens and environmental microorganisms. Professional antigen presenting cells (APCs), such as dendritic cells (DCs), monocytes/macrophages and B lymphocytes, play a crucial role in the recognition of invading pathogens and the initiation of mucosal immune responses (Banchereau and Steinman, 1998; Iwasaki, 2007). To induce an appropriate immune response, these immune cells must migrate from the apical surface of epithelia through the mucosa to the blood and lymph circulation. Important elements affecting immune cell distribution, migration and function are cell-cell and cell-fiber interactions via adhesion molecules and chemokine receptors and their response to chemokine gradients (Springer, 1990; Parlato et al., 2001; Weber et al., 2013). The migration of APCs in response to viral infection in mucosal tissue and the navigation of infected cells to lymph nodes or blood vessels are driven by a complex network of cell signals and interactions. After the virus is exposed to the mucosal epithelium of the upper respiratory tract (URT), epithelial cells immediately recognize viruses via pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and intracellular viral sensors (Akira, 2009; Diamond et al., 2000; Medzhitov, 2001). Cytokines and chemokines, which orchestrate the migration of blood mononuclear cells or mucosal APCs (m-APCs), are also produced during viral infection in the airways (Herold et al., 2006; Vareille et al., 2011; Rudraraju et al., 2013).

Alphaherpesviruses use m-APCs to invade their host (Steukers et al., 2012). Equine herpesvirus type 1 (EHV-1) is an example of an invasive virus of the airway mucosa. The mucosa of the URT is the primary replication site for EHV-1, similar to other alphaherpesviruses (Kydd et al., 1994; Van Maanen, 2002; Gryspeerdt et al., 2010; Vandekerckhove et al., 2010). After initial replication in the epithelial cells, EHV-1 can disseminate through the basement membrane (BM) barrier via a single infected mononuclear immune cell, which then progresses to the blood vessels of the lamina propria and the draining lymph nodes. The virus is detectable in infected m-APCs in equine respiratory explants around submucosal venules at 12 hours post inoculation (hpi) (Kydd et al., 1994; Gryspeerdt et al., 2010; Vandekerckhove et al., 2010). These data suggest that infection with the virus may direct the migration of the m-APCs toward the lymph nodes or blood vessels.

However, it is not clear whether EHV-1 stimulates the migration of these mucosal monocytic cells upon infection or just follows the normal migration of the cells.

In the present study, different types of monocytic cells (blood-derived monocytes, moDCs and nmCD172a$^+$ cells) were placed on top of a polarized nasal mucosal explant and their migration through the mucosa was followed over time. The impact of EHV-1 infection on this transmigration was examined.

2. Materials and Methods

2.1. Collection of tissues and cells

Six horses were used in this study, and each horse constituted an experimental unit. For each experiment blood and nasal samples were collected from the same horse. The horses were of both genders and different ages (3-7 years old). The samples were collected at the slaughterhouse and processed as previously described, with minor modification (Bannazadeh Baghi et al., 2014). In brief, mucosal tissue from the deep intranasal region of the septum was collected and cut into two parts: one was transported to the laboratory in phosphate-buffered saline (PBS), supplemented with 1 µg/ml gentamicin (Gibco), 1 mg/ml streptomycin (Certa), 1000 U/ml penicillin (Continental Pharma), 5 µg/ml fungizone (Bristol-Myers Squibb) and 10% fetal calf serum and the other was transported in the same medium, but without fetal calf serum. To avoid spontaneous migration of DCs, the tissues were kept on ice during transport. The tissue that was transported without serum was cut into small pieces (approximately 50 mm^2) for nasal explant culture. The other sample was used to isolate nmCD172a$^+$ cells.

2.2. Isolation and purification of nmCD172a$^+$ cells

The nasal tissue that was transported in medium containing 10% fetal calf serum was chopped into 3 mm^2 pieces and transferred into medium supplemented with 10 mM EDTA (VWR BDH Prolabo). The sample was incubated at 37°C for 30 minutes with shaking at 250 rpm. Afterwards, the nasal tissue was transferred into medium containing 30 µg/ml DNase I (Sigma-Aldrich) and collagenase type IV (220 U/ml, Gibco, USA) and incubated for 2 hours at 37°C on a shaking platform (Unimax 2010, Heidolph Brinkmann, Germany). The digested cell population was

layered over a Ficoll-Paque™ PLUS gradient (density 1.077; GE Healthcare, Life Sciences), and the interface cell layer was collected. Next, single cell populations were stained with mouse monoclonal anti-CD172a (VMRD, clone DH59B, 1:100, IgG1), and the positive cells were selected by performing MACS with anti-mouse IgG magnetic beads (Miltenyi Biotech Ltd). Immunophenotyping of equine nasal mucosal $CD172a^+$ cells ($nmCD172a^+$ cells) was performed as previously described (Bannazadeh Baghi et al., 2014). The sections were incubated with mouse monoclonal anti-CD172a (VMRD, clone DH59B, 1:100, IgG1), followed by FITC-labeled goat anti-mouse IgG1 (Immunotech) secondary antibody. The cells were analyzed using a FACS Canto flow cytometer (Becton Dickinson Immunocytometry systems). This methodology yielded >90% pure $nmCD172a^+$ cells with >85% viability. Purified $nmCD172a^+$ cells were cultured in 6-well plates.

2.3. Monocyte isolation and DC generation

Equine blood-derived monocytes and monocyte-derived dendritic cells were prepared as previously described, with minor modifications (Dietze et al., 2008). Briefly, monocytes were isolated from 50 ml heparinized blood (100µl heparin). The blood was diluted 1:1 with cold calcium/ magnesium-free phosphate-buffered saline (CMF-PBS), layered onto a Ficoll-Paque™ PLUS gradient (density 1.077; GE Healthcare, Life Sciences) and centrifuged at room temperature for 30 minutes at 2100 rpm. The PBMC interface was washed twice with phosphate-buffered saline and resuspended in RPMI-1640 containing 10% fetal calf serum (FCS), 1 µg/ml gentamycin, 0.1 mg/ml streptomycin and 100 U/ml penicillin. Cells were cultured at 37°C in 5% CO_2 for 1-2 hours. Non-adherent cells were removed from the plate, and adherent cells were maintained in RPMI-1640 medium containing 10% FCS, 1 µg/ml gentamycin, 0.1 mg/ml streptomycin and 100 U/ml penicillin for 24 hours. The moDCs were obtained by adding recombinant equine GM-CSF (Kingfisher Biotech, USA) and IL-4 (R&D Systems, UK) at concentrations of 20 ng/ml and 10 ng/ml, respectively. All cells were collected using accutase detachment solution (Sigma-Aldrich) and counted, and cell viability was assessed by flow cytometry using propidium iodide.

2.4. Monocyte, moDC and nmCD172a$^+$ cell labeling (Cell Tracker™ CFSE)

All prepared cells were resuspended in 1 ml Dulbecco's phosphate-buffered saline (D-PBS) with 0.1% FCS, and the final cell concentration was brought to 4×10^5 cells/ml and incubated at 37°C. Carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) was reconstituted in dimethylsulfoxide (DMSO; BDH, Toronto) just prior to use. Two microliters of 5 mM CFSE solution per milliliter of cells was added to obtain a final working concentration of 10 µM. Cells were incubated for 10 minutes at 37°C, followed by an additional 5 minutes at 4°C. Incubation at the lower temperature allows the dye to label the plasma membrane and slows down endocytosis, reducing dye localization to cytoplasmic vesicles. After CFSE passively diffuses into cells, its acetate groups are cleaved by intracellular esterases to yield a highly fluorescent green signal. CFSE-labeled cells were washed (centrifuged 10 minutes at 4ºC at 1250 rpm) twice with phosphate-buffered saline (PBS) and divided into two equal aliquots for inoculation with EHV-1 or mock-inoculation cells. Both populations were kept in fresh 50% DMEM (Invitrogen), 50% RPMI 1640 (Invitrogen) serum-free medium supplemented with 1 µg/mL gentamicin, 0.1 mg/mL streptomycin and 100 U/mL penicillin. After CFSE labeling, cell viability was above 85%.

2.5. Inoculation of CFSE-labeled cells with EHV-1 and analysis of infection

Labeled monocytes, moDCs and nmCD172a$^+$ cells were inoculated with EHV-1 strain 03P37, which was isolated from the blood of a paralytic horse in 2003 (van der Meulen et al., 2003; Garré et al., 2009). 2×10^5 of cells were inoculated with $10^{6.5}$ $TCID_{50}$ of EHV-1 for 1 hour at 37°C and 5% CO_2. The virus stocks used for inoculation were at the 6th passage: 2 passages in rabbit kidney cells (RK13) and 4 subsequent passages in equine embryonic lung cells (EEL). Inoculated cells were divided into two aliquots (1×10^5 cells). One aliquot was used for the migration assay on tissue, and the other was loaded onto glass inserts in 24-well culture plates (Nunc A/S) to determine the degree of infection. To assess the percentage of infected cells, cells were fixed in 100% methanol at -20°C for 20 minutes and subjected to immunofluorescence staining. Consequently, cells were incubated for 1 hour at 37°C with a rabbit polyclonal anti-IEP antibody (kindly provided by Dr. O'Callaghan, USA, 1:1000) to detect immediate early protein (IEP) expression as an infection marker. The percentage of viral-antigen-positive cells was calculated based on 500 cells from randomly selected microscopic fields. Non-inoculated cells were included as a control. At 0,

24 and 48 hpi, cells were subjected to confocal analysis (Leica TCS SP2 Laser scanning spectral confocal system, Leica microsystems GmbH, Wetzlar, Germany).

2.6. Migration of infected cells in the nasal mucosa

Nasal explants were cut into 50 mm^2 pieces, and each section was placed on 3 ml of agarose (50% sterile 3% agarose (low temperature gelling; Sigma) and 50% 2x medium (50% 2x D-MEM/50% 2x F12)), supplemented with 2 µg/mL gentamicin, 0.2 mg/mL streptomycin and 200 U/mL penicillin in a well of a 6-well culture plate with the epithelium facing upwards. Additional agarose was added until the lateral surfaces of the mucosa were fully occluded. Afterwards, EHV-1 inoculated and mock-inoculated CFSE-labeled monocytes, moDCs and nmCD172a$^+$ cells were resuspended in RPMI-1640 medium with 5% horse hyperimmune serum (HHS) for 1 hour at 37˚C to remove free viruses. Then, the cells were washed with phosphate-buffered saline (PBS) three times and pipetted on to the mucosal explants (1×10^5 cells per explant). To avoid drying out during incubation, the nasal mucosa was covered with a thin film of serum-free medium. Plates were incubated for 0, 24 and 48 hours at 37°C with 5% CO_2. Explants were collected, embedded in methylcellose medium (Methocel® MC, Sigma-Aldrich, St. Louis) and frozen at -70°C. Fifty 10-µm-thick cryosections from each time point were cut and fixed in methanol at -20°C for 20 minutes, and monocytic cells were counted (infected and non-infected). The BM of the tissues was stained with monoclonal mouse anti-collagen VII antibodies (Sigma-Aldrich, St. Louis, 1:300), followed by Texas Red®-labeled goat anti-mouse antibodies (Molecular Probes, 1:100). The nuclei were counterstained with Hoechst 33342 (Molecular Probes) for 10 minutes. As a negative control, 10-µm cryosections of mock-inoculated tissues were stained following the aforementioned protocol. Appropriate isotype-matched control antibodies were used to confirm the specificity of each antibody.

2.7. Statistical analysis

The goal of this experiment was to compare the movement of monocytic cells through the mucosal epithelium at 0, 24 and 48 hours post addition and to analyze the effect of EHV-1 infection. To this end, a completely randomized design (CRD) with three replicates was carried out. Prior to analysis, data were square root transformed according to the number of cells required to satisfy

assumptions of the analysis of variance. Orthogonal contrasts were used to compare different time points in mock-inoculated and EHV-1 inoculated cells. Duncan's multiple range tests were used to compare the means. P values equal to or less than 0.05 were considered statistically significant, and all statistical analyses were carried out using SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

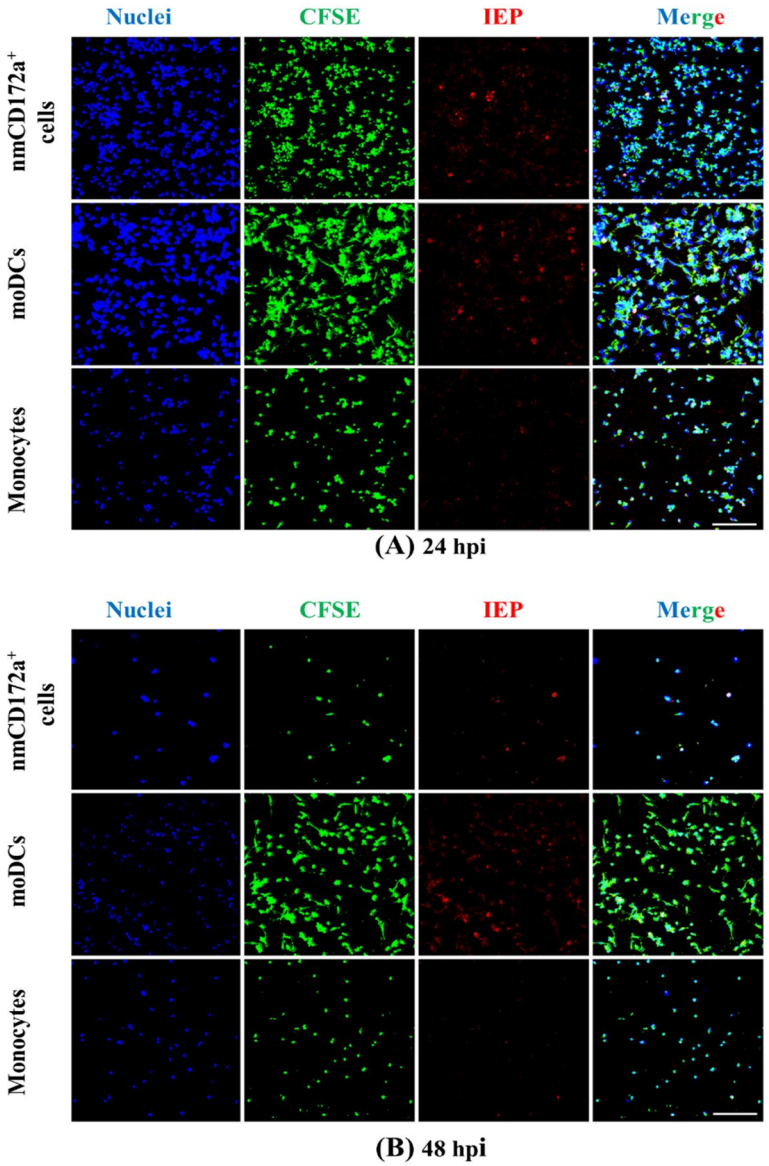

Figure 1. *Immunofluorescence staining of immediate early protein (IEP) in CFSE-labeled nmCD172a$^+$ cells, moDCs and monocytes at 24 (A) and 48 (B) hours post inoculation (hpi). Images are representative of three independent experiments. Scale bar: 100 µm.*

3. Results

3.1. Extent of EHV-1 infection in monocytic cells

Different types of monocytic cells were infected at different levels (Table 1). In total, 13±1.5% and 10±2.5% of moDCs were infected at 24 and 48 hpi, respectively. The percentage of infected nmCD172a$^+$ cells and blood-derived monocytes was slightly lower: 11.5±4.5% and 7±2.5%, and 9±3% and 5±2.5% at 24 and 48 hpi, respectively (Fig. 1). Non-labeled cells were infected to the same degree as labeled cells, demonstrating that CFSE labeling did not influence viral infection.

Table 1. *Percentage of EHV-1-infected (IEP$^+$) cells in different populations of monocytic cells. Data represent the mean ± SD of three independent experiments.*

Cell type	*% IEP-positive cells*	
	At 24 hpi†	*At 48 hpi*
Nasal mucosal CD172a$^+$ cells	*11.5 ± 4.5*	*9 ± 3*
Monocyte-derived dendritic cells	*13 ± 1.5*	*10 ± 2.5*
Monocytes	*7 ± 2.5*	*5 ± 2.5*

† hpi = hours post inoculation

3.2. Migration of monocytic cells across the nasal mucosal epithelia toward the submucosa

To determine whether monocytes, moDCs and nmCD172a$^+$ cells cross the nasal epithelium, polarized, agarose-embedded nasal mucosa explants were utilized. To analyze the migratory behavior of monocytic cells, CFSE-labeled cells were layered on top of the mucosa, and explants were collected at 0, 24 and 48 hours. Afterwards, cryosections were cut and stained for BM and cell nuclei. Four regions of interest were defined: A-mucosa surface, B-epithelium, C-basement membrane + 50 µm underneath (lamina propria), and D-submucosa (Fig. 2). At 0 hours, cells were only observed on top of the mucosa surface. There was no significant difference in the migration of cells between the 24- and 48-hour intervals. The number of monocytic cells varied at different levels of the nasal explant. A small number (1%-7%) of CFSE-labeled moDCs and nmCD172a$^+$ cells were detected in the lamina propria and submucosa, consistent with the assumption that

monocytic cells migrate toward the draining lymph and blood vessels in the lamina propria and submucosa under steady-state conditions. Blood-derived monocytes labeled with CFSE were not observed in the deep layers of the lamina propria and submucosa.

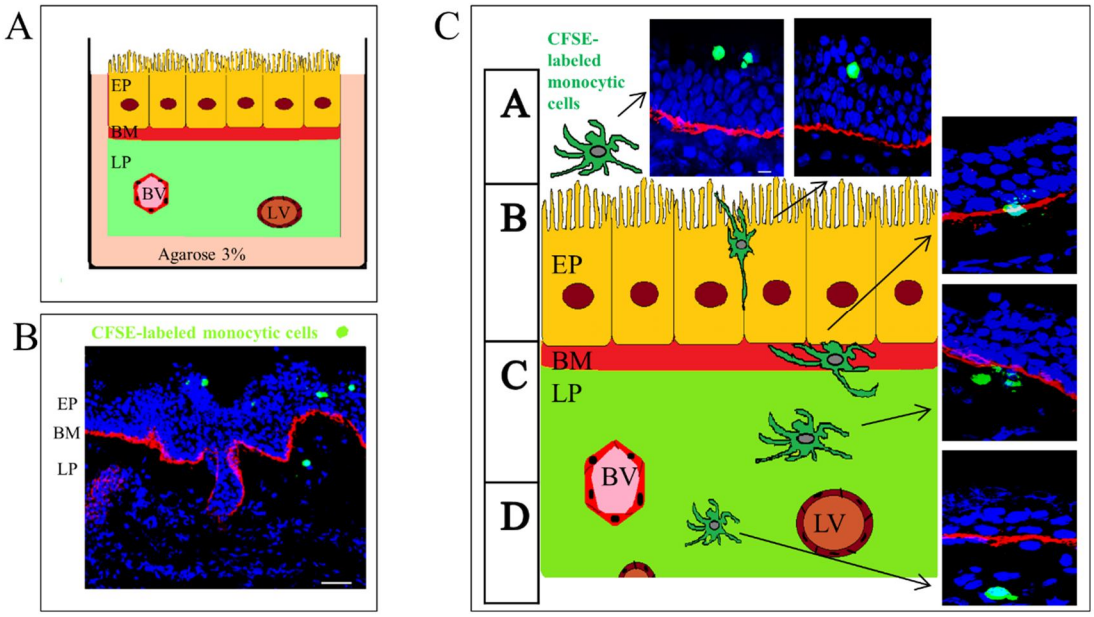

Figure 2. *A schematic model of the polarized tissue explant system used to study the migration of monocytic cells in nasal mucosal explants (A). All cell types (nmCD172a$^+$ cells, moDCs and monocytes) were labeled with 10 µM CFSE for 10 min. Then, cells were added to the mucosal surfaces of polarized explants at 1×10^5 cells per explant (50 mm^2). After 0-, 24- and 48-hour time periods, the tissue explants were fixed and sectioned (B); scale bar: 50 µm. Regions of interest were divided into four levels (C); scale bar: 10 µm. Representative confocal photomicrographs illustrate the migration of monocytic cells (green) in the epithelium (EP) and lamina propria (LP). BM: basement membrane, BV: blood vessel, LV: lymph vessel.*

Table 2. *Quantification and orthogonal contrasts of mock-inoculated and EHV-1-inoculated monocytic cells at different levels in the nasal mucosa explant. Fifty sections were made, and the total number and average percentage of migrating cells ($CFSE^+$ / $CFSE^+$ IEP^+) per zone and time point were determined.*

Cell type	Regions of interest	Total number (and %†) of mock-inoculated cells $CFSE^+$ in 50 sections at … hours post addition		Total number (and %) of EHV-1 inoculated cells $CFSE^+$ (III & V) / $CFSE^+$ IEP^+ (IV & VI) in 50 sections at … hours post addition	
		24 (I)	48 (II)	24 (III) / 24 (IV)	48 (V) / 48 (VI)
nmCD172a$^+$ cells	A-mucosa surface	187 ± 91 (76%)	200 ± 87 (77%)	228 ± 149 (82%) / 27 ± 19 (78%)	163 ± 61(79.5%) / 25 ± 6 (71%)
	B-epithelium	44 ± 19 (18.5%)	42 ± 31 (17.5%)	33 ± 7 (15%) / 8 ± 5 (22%)	32 ± 20 (16%) / 9 ± 3 (25.5%)
	C-basement membrane + lamina propria	16 ± 5 (5%)	9 ± 1 (3.5%)	2 ± 1 (1.5%) / 0 ± 0 (0%)	4 ± 3 (3.5%) / 2 ± 1 (3.5%)
	D-submucosa	1 ± 1 (0.5%)	4 ± 2 (2%)	3 ± 1 (1.5%) / 0 ± 0 (0%)	1 ± 0 (1%) / 0 ± 0 (0%)
moDCs	A-mucosa surface	227 ± 113 (74%)	192 ± 120 (76%)	156 ± 42 (84%) / 17 ± 5 (79%)	118 ± 65(75.5%) / 19 ± 9 (64.5%)
	B-epithelium	58 ± 31 (19%)	42 ± 28 (16.5%)	25 ± 6 (13%) / 4 ± 2 (19%)	28 ± 15 (18%) / 8 ± 5 (26%)
	C-basement membrane + lamina propria	20 ± 15 (6%)	16 ± 11 (6.5%)	4 ± 2 (2.5%) / 1 ± 1 (2%)	8 ± 5 (5%) / 3 ± 1 (9.5%)
	D-submucosa	3 ± 1 (1%)	2 ± 1 (1%)	1 ± 1 (0.5%) / 0 ±0 (0%)	2 ± 0 (1.5%) / 0 ± 0 (0%)
Monocyte	A-mucosa surface	226 ± 80 (91%)	202 ± 68 (88%)	203 ± 21(92%) / 14 ± 3 (88%)	201 ± 142 (91%) / 14 ± 5 (89%)
	B-epithelium	21 ± 4 (8.5%)	27 ± 12 (11.5%)	18 ± 2 (8%) / 2 ± 1 (12%)	19 ± 4 (8.5%) / 2 ± 1 (11%)
	C-basement membrane + lamina propria	1 ± 1 (0.5%)	1 ± 1 (0.5%)	0 ± 0 (0%) / 0 ± 0 (0%)	1 ± 1 (0.5%) / 0 ± 0 (0%)
	D-submucosa	0 ± 0 (0%)	0 ± 0 (0%)	0 ± 0 (0%) / 0 ± 0 (0%)	0 ± 0 (0%) / 0 ± 0 (0%)

† % = Average percentage of the migrated cells. I vs II (F value = 0.05 non significant (ns), P value = 0.081 ns), III vs V (F value = 0.02 ns, P value = 0.088 ns), I vs III (F value = 0.68 ns, P value = 0.40 ns), II vs V (F value = 0.56 ns, P value = 0.45 ns), IV vs VI (F value = 0.05 ns, P value = 0.082 ns), I & II vs III & V (Fvalue = 0.02 ns, P value = 0.087 ns).

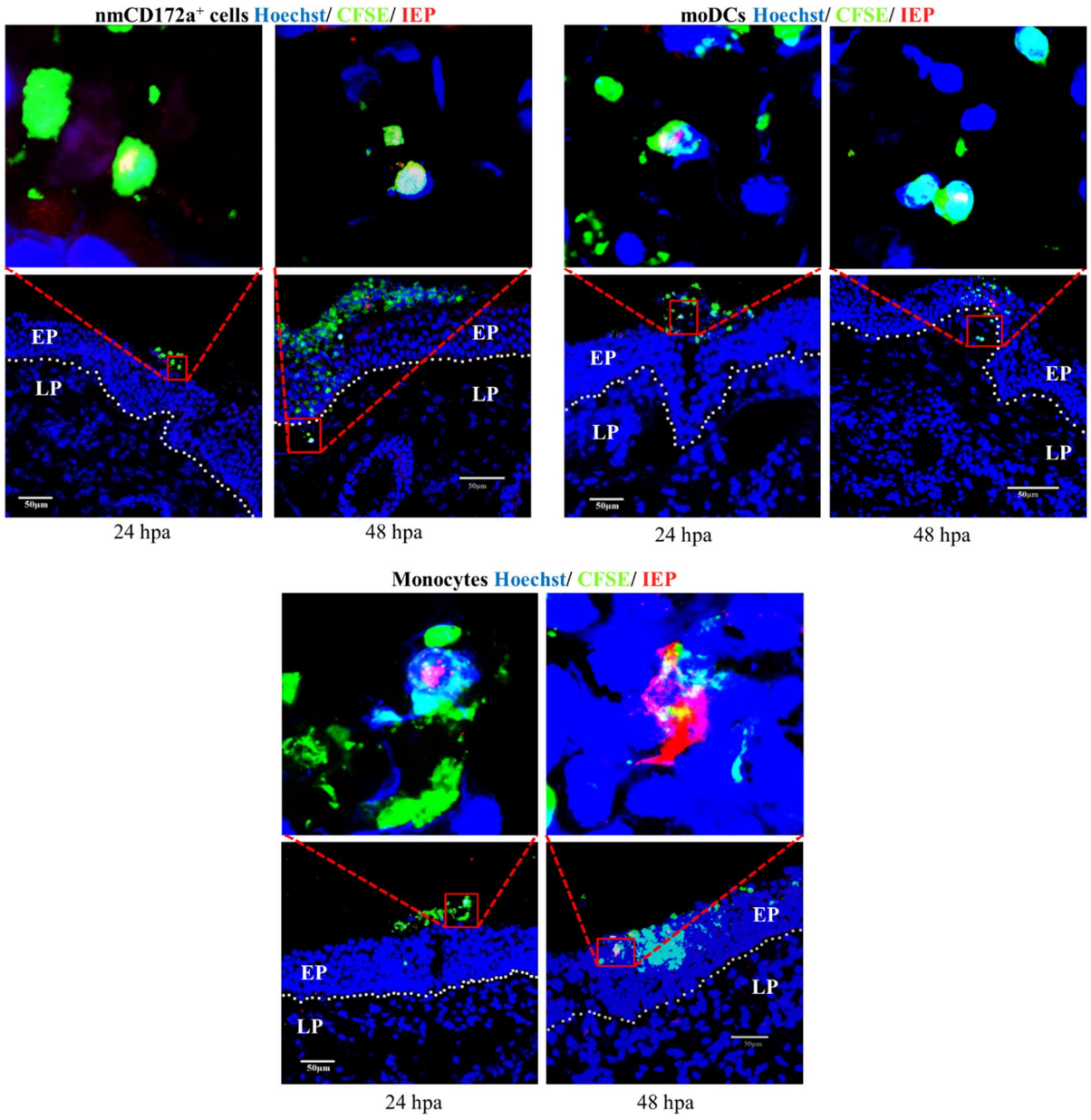

Figure 3. *Representative fluorescence microscopy images of nasal tissues exposed to EHV-1-inoculated monocytic cells. Labeled and EHV-1 (strain 03P37) inoculated cells (nmCD172a+ cells, moDCs and monocytes) were added to the mucosal surfaces of nasal explants (1×105 cells per explant, 50 mm2). After 0, 24 and 48 hours post addition (hpa), the tissue explants were fixed, sectioned and stained. Then, the sections were examined for CFSE-labeled cells (green) and for IEP (red). EHV-1-infected (IEP+) monocytic cells (CFSE+) were identified by the colocalization of red and green (yellow). White lines indicate the border between the lamina propria and the mucosal epithelium. Images are representative of explants from three independent horses; original scale bar, 50 µm.*

3.3. EHV-1 infection did not change the migratory behavior of monocytic cells

To study the migration pattern of EHV-1-infected cells, each cell type was labeled with CFSE prior to inoculation. Mock-inoculated labeled cells were included as control. The EHV-1 infection status was assessed using IEP. All mock-inoculated ($CFSE^+$ IEP^-) and EHV-1 inoculated ($CFSE^+$ IEP^+ /$CFSE^+$ IEP^-) monocytic cells per zone and time point are shown in Table 2. Both EHV-1-inoculated and mock-inoculated monocytic cells migrated into the epithelium and lamina propria. There was no significant difference in migration between the mock-inoculated and EHV-1-inoculated cells at 24 or 48 hours. Analysis of the colocalization between CFSE and IEP ($CFSE^+$ IEP^+) revealed that over 80% of the infected nmCD172a$^+$ cells and moDCs remained on top of the epithelium after 24 hours. The percentage of these cells decreased to approximately 70% on the surface of the nasal mucosa after 48 hours. More than 85% of the $CFSE^+$ IEP^+ blood-derived monocytes remained on the surface of the nasal mucosa after 24 and 48 hours. Almost 2% and 9% of the EHV-1-infected and CFSE-labeled moDCs penetrated through the BM at 24- and 48-hour intervals, while only 0% and 3% of the infected nmCD172a$^+$ cells ($CFSE^+$ IEP^+) crossed the BM. Infected blood-derived monocytes were never observed under the BM (Fig. 3). The number of EHV-1-infected ($CFSE^+$ IEP^+) nmCD172a$^+$ cells and moDCs that transmigrated into the nasal tissue was significantly ($P < 0.05$) higher than that of blood-derived monocytes after 48 hours (Table 3), suggesting that moDCs and nmCD172a$^+$ cells are better transporters at the mucosal epithelium compared to blood monocytes.

Table 3. *Comparison of the mean number of EHV-1-infected ($CFSE^+$ IEP^+) monocytic cells that transmigrated into the nasal tissue after 24 and 48 hpa.*

Cell type	*Number of $CFSE^+$ IEP^+ transmigrated cells into the epithelium at 24hpa*	*Number of $CFSE^+$ IEP^+ transmigrated cells into the epithelium at 48hpa*
nmCD172a$^+$ cells	*2.4894^a*	*2.7462^a*
moDCs	*2.1838^{ab}*	*2.5480^a*
Monocyte	*1.8821^b*	*1.9154^b*

Data underwent square root transformation.

Values within a column followed by different letters are significantly different at the 0.05 level using the Duncan test. Values within a column followed by the same letters are not significantly different using the Duncan test.

4. Discussion

Epithelial cells of the respiratory mucosa are the primary target cells for many pathogens, especially viruses. The ability of APCs to migrate throughout the mucosal epithelium is a critical aspect of their immunological function and could be an essential factor for the mucosal immune response against invasive pathogens. These immune cells form a network within mucosal surfaces to capture antigens, interact with each other, migrate to the draining lymph node and present antigens to T cells (Steinman and Banchereau, 2007). The pathogenesis of equine herpesvirus type 1 (EHV-1) infection starts with the invasion of the virus into the epithelium of the upper respiratory tract (URT) and to deeper tissue to initiate cell-associated viremia. Mucosal APCs (m-APCs) act as a "Trojan horse" for the virus because they can migrate through the mucosal epithelium and eventually to the circulatory system without being recognized by the immune system (Kydd et al., 1994; Van Maanen, 2002; Gryspeerdt et al., 2010; Vandekerckhove et al., 2010). The present study was designed to evaluate the migration of monocytic cells in the URT and to determine their importance for EHV-1 transmigration. To this end, we generated a polarized nasal mucosa explant system. Agarose was used to cover the bottom and the lateral edges of the explant, leaving only the apical mucosal surface accessible to cell penetration. This system allowed us to study the motility of monocytic cells alone and the transmigration of these cells during EHV-1 infection within nasal mucosal explants.

First, we examined the movement of normal, non-virus-inoculated monocytic cells. Second, we tracked EHV-1-inoculated monocytic cells in the nasal mucosa to assess the effect of infection on the migratory behavior of these cells. At 0 hours, the three monocytic cell types were solely detected on top of the mucosal epithelium. Afterwards, a percentage of cells (16-26%) invaded into the mucosa between 24 and 48 hours. Why the transmigration was restricted to a certain subpopulation is not clear, but we plan to examine this in the near future. In contrast with moDCs and nmCD172a$^+$ cells, blood-derived monocytes did not migrate into deep layers of the lamina propria and submucosa, which is consistent with their normal behavior. Blood monocytes mainly migrate from vessels toward the mucosal epithelium en route to the lymph and blood circulation, not from the apical side of the epithelium toward the basolateral side. A subset of blood monocytes leaves the blood circulation, differentiates into macrophages or DCs and patrols healthy tissues, including the mucosal epithelium of the respiratory tract (Imhof and Aurrand-Lions, 2004; Auffray

et al., 2007; Yang et al., 2014; Randolph et al., 1999). Macrophages destroy pathogens. DCs capture antigens and transport them to the draining lymph nodes and blood circulation and present antigens to the immune system (Auffray et al., 2007; Jakubzick et al., 2013). In the present study, the role of moDCs and nmCD172a$^+$ cells as potential transport vehicles was illustrated by their migration into mucosal tissue. We observed that 16-26% of cells crossed the nasal epithelium, and 1-7% migrated through the lamina propria and submucosa. These results agree with the well-recognized migratory behavior of mucosal macrophages and DCs, which migrate to draining lymph nodes and present antigens to naive T cells (Coombes and Powrie, 2008; Tugizov et al., 2012; Kissenpfennig et al., 2005). Thus, DCs and nmCD172a$^+$ cells are more agile compared to other blood and tissue mononuclear cells.

Next, we examined the migration pattern of EHV-1-inoculated monocytic cells. The three types of monocytic cells were infected to different degrees. The percentage of infected (IEP positive) cells was higher in moDCs (13±1.5% at 24 hpi and 10±2.5% at 48 hpi) compared to nmCD172a$^+$ cells (11.5±4.5% at 24 hpi and 9±3 at 48 hpi) and blood-derived monocytes (7±2.5% at 24 hpi and 5±2.5% at 48 hpi). Both EHV-1-inoculated (CFSE$^+$ IEP$^+$/IEP$^-$) and mock-inoculated (CFSE$^+$ IEP$^-$) monocytic cells migrated into the epithelium and lamina propria to a similar extent. The number of EHV-1-infected (CFSE$^+$ IEP$^+$) moDCs and nmCD172a$^+$ cells that passed through the nasal epithelium was significantly higher than EHV-1-infected blood-derived monocytes after 48 hours. These data are consistent with previous observations on the transmigration of HIV-infected macrophages through the paracellular space of intact mucosal epithelia and paracellular space of polarized endometrial epithelial cells (Anderson et al., 2010; Carreno et al., 2002). Moreover, HIV-infected macrophages can transmigrate across intestinal and fetal oral mucosal epithelia (Tugizov et al., 2012). These macrophages use adhesion molecules to stay attached to epithelial cells, where they perform their antigen uptake and surveillance functions. Once macrophages are activated, they utilize cell junctions to migrate back into the tissue. Although infected moDCs and nmCD172a$^+$ cells penetrated through the epithelial cells, only moDCs could transfer EHV-1 through the BM barrier at 24 hours. After 48 hours, nmCD172a$^+$ cells were able to carry EHV-1 through the BM barrier but to a much lesser extent than moDCs. Considerable evidence indicates that DCs are actively involved in the transmigration of pathogens from mucosal surfaces toward local lymph nodes. Many pathogens utilize mucosal immune cells and DCs for transport and transfer to target cells. Human Immunodeficiency Virus (HIV) is a well-known virus that hijacks

immature DCs and transfers them from the apical side of epithelium into the lamina propria (Cunningham et al., 2013; Harman et al., 2013). Tugizov et al. reported that monocytes/macrophages/Langerhans cells transfer Epstein-Barr virus (EBV), a human gammaherpesvirus, into the oral mucosal epithelium and spread the virus within the body (Tugizov et al., 2007). Therefore, a similar mechanism might take place in our model system of EHV-1 transmigration through mucosal epithelium. Blood-derived monocytes appear to take up viruses but fail to breach the BM barrier. This finding is likely due to the inability of these cells to differentiate into antigen presenting cells (Jakubzick et al., 2013; Palframan et al., 2001). Both moDCs and nmCD172a$^+$ cells can capture EHV-1 and provide a path for the virus to penetrate intact mucosal epithelia. Thus, moDCs and nmCD172a$^+$ cells may operate as viral transport vehicles to local lymph nodes and internal organs via blood circulation.

In conclusion, our findings illustrate the behavior and function of different monocytic cells within the mucosal tissue. These cells become infected with EHV-1 at the respiratory mucosa, the initial site of the infection, and transport of the virus from the apical side of the epithelium into the lamina propria. Our data demonstrate that monocytic cells clearly play a role in the pathogenesis of EHV-1 infection. Nevertheless, further studies are needed to confirm this invasion pathway and to identify other immune cells and molecules involved in this process. Understanding the fundamental aspects of EHV-1 entry during equine upper respiratory tract infection remains a critical step for preventing EHV-1 infection and developing further vaccinal strategies and virucidal agents.

Conflict of interest statement

The authors declare no conflicts of interest.

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CHAPTER IV

Effect of an equine herpesvirus type 1 (EHV-1) infection of the nasal mucosa epithelial cells on integrin alpha 6 and on different components of the basement membrane

Hossein Bannazadeh Baghi and Hans J. Nauwynck

Manuscript in preparation

Summary

The respiratory mucosa is the common port of entry of equine herpesvirus type 1 (EHV-1) and several other alphaherpesviruses. An important prerequisite for successful host invasion of the virus is to cross the epithelial cell layer and the underlying basement membrane barrier. In the present study, an analysis was performed to see if an EHV-1 infection of nasal mucosa epithelial cells leads to damage of the underlying basement membrane. A detailed quantitative analysis system was set up to determine the impact of the virus replication on different components of the basement membrane. Nasal mucosa explants were inoculated with EHV-1 and collected at 0, 24 and 48 hours post inoculation (hpi). Then, double immunofluorescence stainings were performed to detect viral antigen positive cells on the one hand and integrin alpha 6 (ITGA6), laminin, collagen IV and collagen VII on the other hand. The thickness of integrin alpha 6, laminin, collagen IV and collagen VII was measured in regions of interest (ROI) at a magnification of 200X by means of the software imaging system ImageJ. Regions of interest were defined underneath non-infected and infected regions. In non-infected regions 22 - 28 % of the ROI was stained for integrin alpha 6, 18 - 37 % for laminin, 14 - 38 % for collagen IV and 18 - 26 % for collagen VII. In infected regions this percentage was significantly decreased for integrin alpha 6 to 0.1 - 9 % and 0.1 - 6 % after 24 and 48 hours of inoculation, respectively. Infection did not alter the percentages for laminin and collagen IV. For collagen VII an increase in the percentage (from 18 - 26 % to 28 - 39 %) could be observed underneath EHV-1-infected plaques at 48 hours of inoculation. In conclusion, the results revealed a substantial impact of EHV-1 infection on integrin alpha 6 and collagen VII, two important components of the basement membrane barrier.

1. Introduction

Equine herpesvirus type 1 (EHV-1) is a ubiquitous alphaherpesvirus and a causative agent of different disease symptoms in horses including epidemic respiratory disease, abortion, neonatal foal death, equine herpesvirus myeloencephalopathy (EHM), and chorioretinopathy (Allen et al., 2004; Dunowska, 2014). Despite vaccination, EHV-1 remains a constant threat to horses worldwide, mainly because the immune responses induced after both infection and vaccination are not fully protective (Bürki et al., 1990). The initial infection starts with replication of EHV-1 in the epithelial cells lining the airway mucosa. EHV-1 does not only have a tropism for epithelial cells, but also targets monocytic cells and T-lymphocytes, hijacking these cells for transport across the basement membrane barrier (Kydd et al., 1994; Vandekerckhove et al., 2010; Gryspeerdt et al., 2010; Bannazadeh Baghi and Nauwynck, 2014). At present it is not known by which mechanism the basement membrane is crossed by EHV-1-infected immune cells. This is the case since there is a discrepancy between the permeability of virus plaques and infected cells across the basement membrane.

The basement membrane is an amorphous, dense, highly cross-linked and sheet-like structure of 50 - 100 nm in thickness that separates cells from the underlying lamina propria and provides a structural support for most epithelia (Kalluri, 2003; Kelley et al., 2014). The basement membrane is defined biochemically by its typical components and morphologically by its characteristic appearance in electron micrographs which consists of two thin structural layers. The first layer, the basal lamina, is synthesized by epithelial cells and the second layer, the reticular lamina, is made by fibroblasts. The basal lamina is subdivided into a clear lamina lucida directly under the epithelial cells and a structurally opaque lamina densa (Evans et al., 1990; Evans et al., 2010b). The main components of the basement membrane are: laminins, type IV collagen, nidogen and the heparan sulfate proteoglycans (HSPGs), and also often agrin, fibulins, fibronectin and other types of collagen (I, III, V, VI, VII, and XVIII) as well as various integrins, which are plasma membrane anchoring proteins that act as a ligand of basement membrane components (Erickson and Couchman, 2000; Yurchenco et al., 2004; Yurchenco and Patton, 2009). This extracellular matrix forms a barrier underneath the layer of mucosal epithelial cells and contributes to their filter function of selecting or permitting the passage of diverse molecules across the corresponding barrier (Rippe and Davies, 2011; Ockleford et al., 2013; Mestres et al., 2014). Potential

mechanisms for immune cell transmigration through the mucosal basement membrane include: transmigration of cells through preformed holes or incisions, proteolytic digestion of extracellular matrix components and non-proteolytic force-dependent mechanisms (Rowe and Weiss, 2008; Sorokin, 2010). The mechanism by which free viruses or viral infected cells breach the basement membrane, in order to invade deeper tissues and thus spread throughout the body, is largely unexplored. In the present study the effect of an EHV-1 infection on integrin alpha 6, laminin, collagen IV and collagen VII of the nasal mucosal basement membrane barrier was examined.

2. Materials and Methods

2.1. Animals and nasal tissue collection

Nasal mucosa was collected from horses in the slaughterhouse and was used to obtain nasal explants. Horses negative for nasal/ocular discharge and lung pathology were selected. All horses were between 4 and 8 years old and seropositive for EHV-1. Nasal explants of three individual horses were collected. The tissues were transported in phosphate-buffered saline (PBS), supplemented with 1 µg/ml gentamicin (Gibco), 1 mg/ml streptomycin (Certa), 1000 U/ml penicillin (Continental Pharma), and 5 µg/ml fungizone (Bristol-Myers Squibb) to the laboratory. Then, tissues were cut into strips for nasal explant culture.

2.2. Cultivation of the nasal mucosal explants

The cultivation of nasal mucosal explants was performed with minor modification as previously described (Bannazadeh Baghi and Nauwynck, 2014). In brief, mucosal explants were stripped from the surface by the use of surgical blades (Swann-Morton). Then, the stripped mucosa was divided into equal explants of 25 mm^2 and placed in six-well plates with the epithelial side facing up on a fine-meshed gauze and cultured for 24 h (37 °C, 5% CO_2) at an air-liquid interface with serum-free medium containing 50% DMEM (Invitrogen), 50% Ham's F-12 GlutaMAX (Invitrogen) and supplemented with 0.1 mg/mL gentamicin (Invitrogen), 0.1 mg/mL streptomycin (Certa), 100 U/mL penicillin (Continental Pharma) and 0.01 mg/mL fungizone (Bristol-Myers Squibb, USA). For virus inoculation, the tissue explants were transferred from the gauzes on 3 ml of agarose (50% sterile 3% agarose (low temperature gelling; Sigma) and 50% 2x medium (50% 2x D-MEM/50% 2x F12)), supplemented with 2 µg/mL gentamicin, 0.2 mg/mL streptomycin and

200 U/mL penicillin in a well of a 6-well culture plate with the epithelium facing upwards. Additional agarose was added until the lateral surfaces of the mucosa were fully occluded. Only a thin film of serum-free medium (50% Roswell Park Memorial Institute medium (RPMI, Invitrogen) /50% Dulbecco's Modified Eagle Medium (DMEM, Invitrogen)) supplemented with 0.3 mg/mL glutamine (BHD Biochemical), 1 µg/mL gentamycin (Invitrogen), 0.1 mg/mL streptomycin (Certa) and 100 U/mL penicillin (Continental Pharma)) covered the explants, thereby mimicking the air/liquid interface found in the respiratory tract of the living animal. Explants were maintained at 37°C in an atmosphere containing 5% CO_2.

2.3. Virus

EHV-1 strain 03P37, which was isolated from the blood of a paralytic horse in 2003, was used in this study (van der Meulen et al., 2003; Garré et al., 2009). The virus stocks used for inoculation were at the 6^{th} passage: 2 passages in rabbit kidney cells (RK13) and 4 subsequent passages in equine embryonic lung cells (EEL).

2.4. Virus inoculation and sample collection of nasal explants

Explants were inoculated with 500 µL of EHV-1 at a titer of $10^{6.5}$ $TCID_{50}$/mL for 1 hour at 37 °C in the presence of 5% CO_2. Afterwards, they were washed three times with medium and further incubated for 0, 24 and 48 hours at 37°C with 5% CO_2. To avoid drying out during incubation, the nasal mucosa was covered with a thin film of serum-free medium. Explants were collected, embedded in methylcellose medium (Methocel® MC, Sigma-Aldrich, St. Louis) and frozen at -70°C. Fifty 12-µm-thick cryosections from each time point were cut and fixed in methanol at -20°C for 20 minutes.

2.5. Immunofluorescence staining

Viral proteins were stained by incubation with biotinylated equine polyclonal anti-EHV-1 IgG (van der Meulen *et al.*, 2003), followed by streptavidin-Texas Red® (Molecular Probes, Eugene, OR). Subsequently, the basement membrane components of the tissues were stained separately with monoclonal mouse anti-integrin alpha 6 antibodies (IgG1, Santa Cruz Bio), polyclonal rabbit anti-laminin antibodies (IgG, ab11575; Abcam), goat anti-collagen IV antibodies (IgG, Southern

Biotech) and monoclonal mouse anti-collagen VII antibodies (IgG1, Sigma-Aldrich, St.Louis), followed by secondary FITC-labeled goat anti-rabbit antibodies, Alexa Fluor® 488 rabbit anti-goat IgG, FITC-labeled goat anti-mouse antibodies (Molecular Probes, Eugene, OR). All antibodies were diluted in PBS (1:100) and incubated for 1 hour at 37°C and 5% CO_2. The nuclei were counterstained with Hoechst 33342 (Molecular Probes) for 10 minutes. As a negative control, 12-µm cryosections of mock-inoculated tissues were stained following the aforementioned protocol. Appropriate isotype-matched control antibodies were used to confirm the specificity of each antibody. All stainings were analyzed with a confocal laser scanning microscope (Leica TCS SPE laser scanning spectral confocal system, Leica Microsystems GmbH, Wetzlar) and the Leica confocal software.

2.6. Basement membrane analysis

To quantify the basement membrane components under infected (plaques) and non-infected areas, a double immunofluorescence staining was performed. By using the software imaging system ImageJ, the percentage of pixels positive for integrin alpha 6, laminin, collagen IV and collagen VII was measured in standardized regions of interest (50 x 50 pixels) in sections at a magnification of 200X (Figure 1).

2.7. Statistical analysis

The data were statistically evaluated by analysis of variance (ANOVA) using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA). Results are shown represent mean + SD of three independent experiments with three different horses (*$P < 0.05$; **$P < 0.01$; ns, not significant).

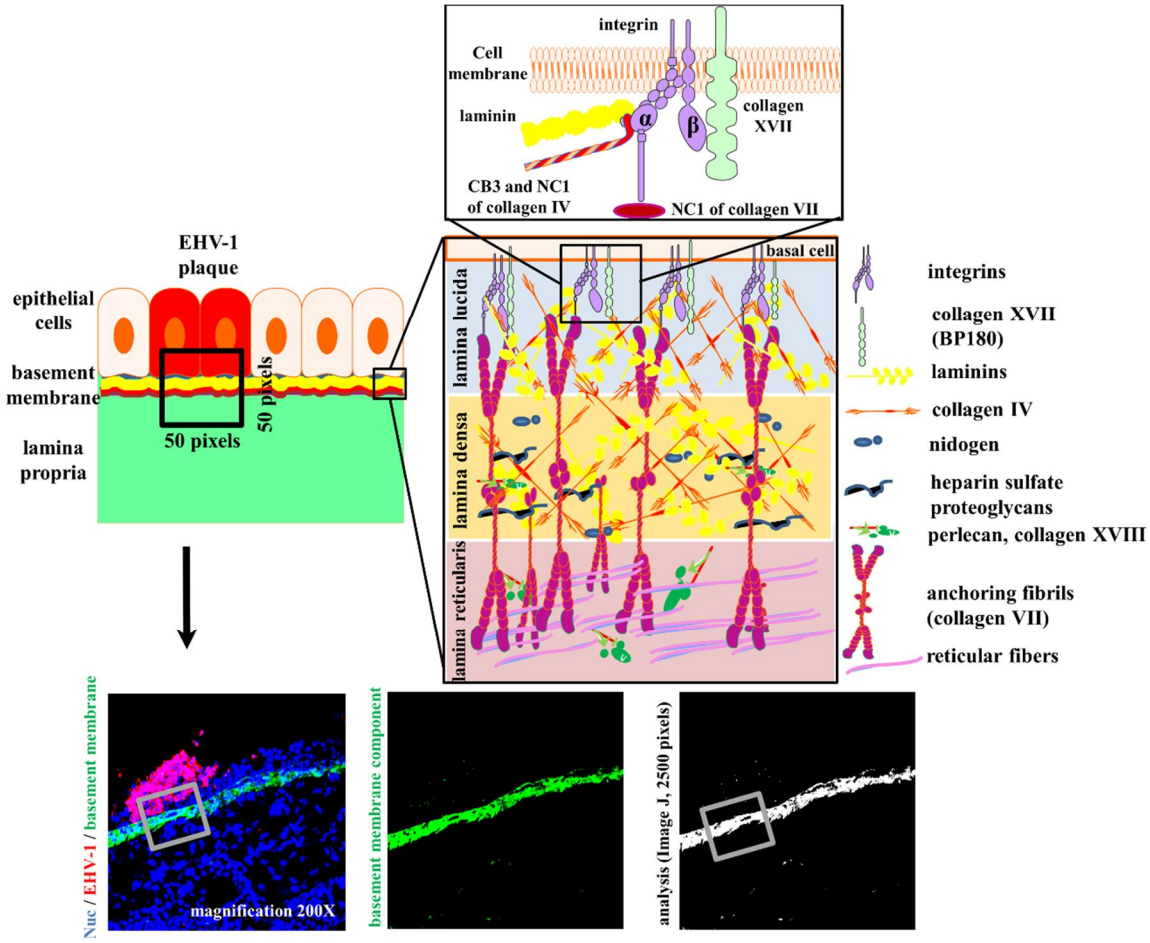
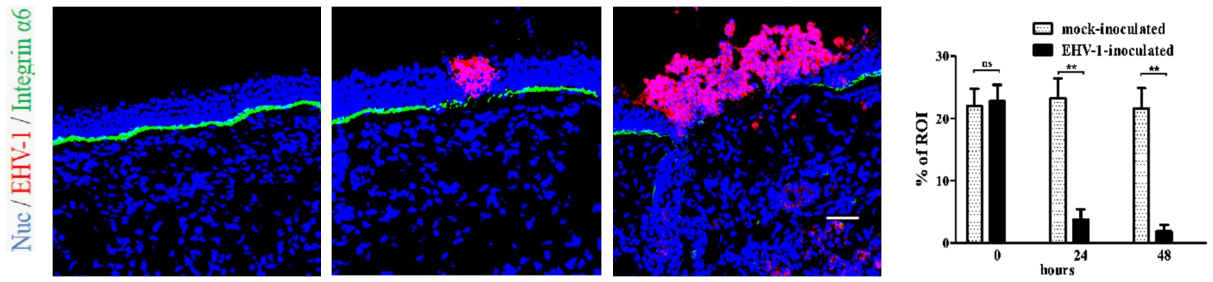


Figure 1. *Schematic overview of the nasal mucosal basement membrane with its main components. The regions of interest (50 × 50 pixels) were set in sections at a magnification of 200X and the percentage of pixels positive for different components of the basement membrane were measured by using the software imaging system ImageJ.*

3. Results

3.1. Integrin alpha 6 (ITGA6)

Integrin alpha 6 is present as a full lining underneath the epithelial cells of mock-inoculated nasal mucosal tissues: 22 - 28 % in a square of 2500 pixels (region of interest, ROI). Upon inoculation with EHV-1, integrin alpha 6 specifically disappeared underneath the EHV-1 positive plaques: 0.1 - 9 % at 24 hpi and 0.1 - 6 % at 48 hpi (Figure 2).

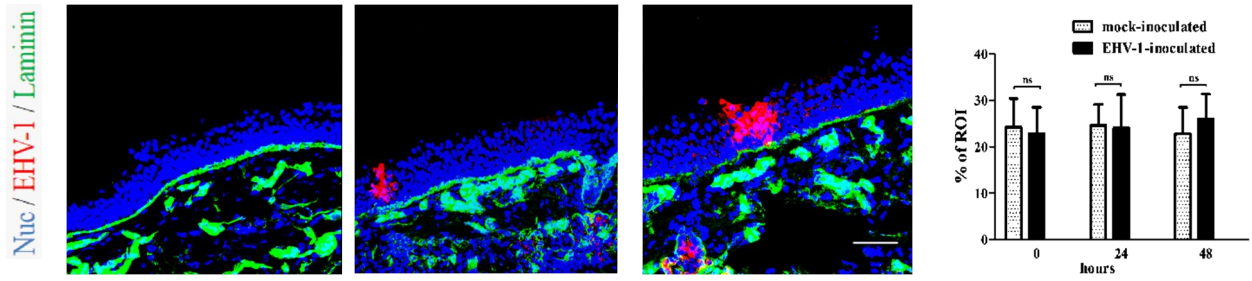


0 hpi 24 hpi 48 hpi

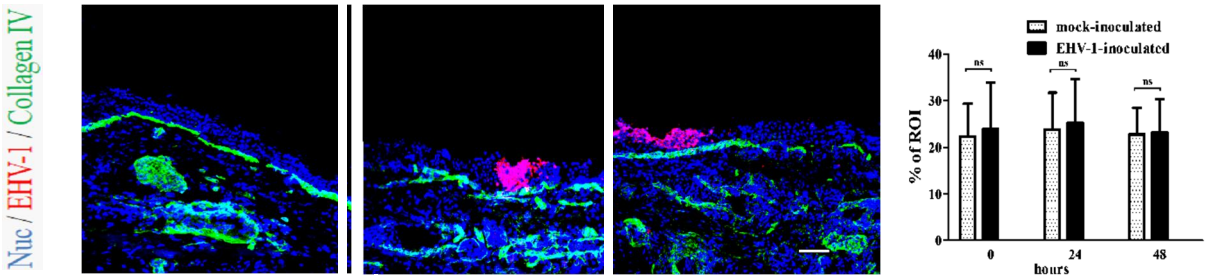
Figure 2. *Confocal photographs illustrating the lining of integrin alpha 6 (green) and the effect of EHV-1 infection in the nasal mucosa epithelial cells (red) at 0, 24 and 48 hours post inoculation (hpi). Scale bar: 50 µm. EHV-1 infection in the epithelial cells clearly damaged integrin alpha 6 (**P < 0.01).*

3.2. Laminin and Collagen IV

With antibodies against laminin and collagen IV a strong obvious staining was detected underneath the epithelial cells of mock-inoculated explants and underneath a plaque of EHV-1 infected cells. The thickness of the laminin and collagen IV underneath an EHV-1 plaque at 24 and 48 hpi was similar to that of the laminin and collagen IV at 0 hpi and non-inoculated tissues (Figure 3).



0 hpi 24 hpi 48 hpi

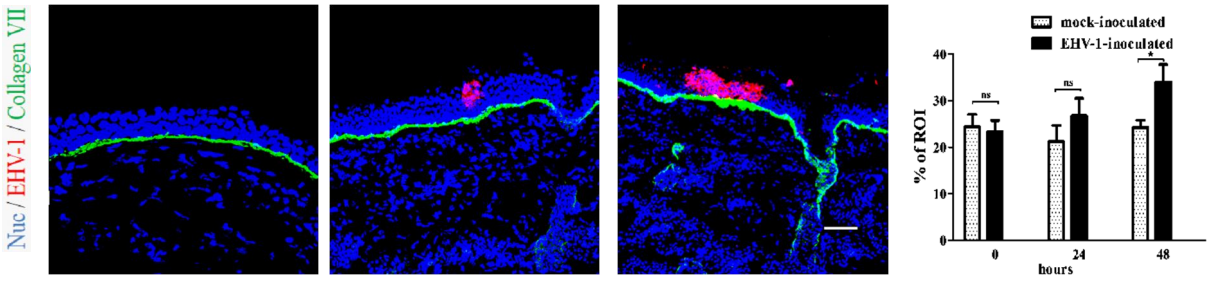


0 hpi 24 hpi 48 hpi

Figure 3. *Confocal photographs illustrating the laminin and collagen IV of basement membrane (green) and plaques of EHV-1-infected cells (red) at 0, 24 and 48 hpi. Scale bar: 50 µm. EHV-1 infection had no impact on laminin and collagen IV.*

3.3. Collagen VII

Monoclonal antibodies against collagen VII revealed a full lining underneath the epithelial cells. Collagen VII thickness was not significantly altered at 24 hpi (Figure 5). However, the thickness of collagen VII was significantly ($P < 0.05$) increased below plaques of EHV-1 infected cells (28 - 39 % in a square of 2500 pixels) after 48 hpi compared to mock-inoculated tissues (18 - 26 %). Collagen VII thickness was not significantly altered at 24 hpi (Figure 5).



0 hpi 24 hpi 48 hpi

Figure 5. *Confocal photographs illustrating the collagen VII of the basement membrane (green) and plaques of EHV-1-infected cells (red) at 0, 24 and 48 hpi Scale bar: 50 µm. An EHV-1 infection caused thickening of the area containing collagen VII after 48 hpi (*P < 0.05).*

4. Discussion

The airway epithelium constitutes the first barrier of defense against environmental microorganism by providing not only a mechanical and chemical barrier to impede entry of foreign particles, but also by its ability to orchestrate both the innate and adaptive immune responses. The basement membrane forms a layer underneath the respiratory epithelial cells which plays an important role in the maintenance of mucosal tissue architecture and orchestrates the homing of immune cells as well as tissue regeneration during pathological events (Timpl, 1989; Yurchenco, 2011). Mucosal basement membrane components are attractive targets for adherence and invasion by various microorganisms. Equine herpesvirus type 1 (EHV-1) is an invasive virus of the respiratory mucosa. After initial replication in the epithelial cells, EHV-1 can cross the basement membrane barrier via single infected mononuclear immune cells, which then progress to the blood vessels of the lamina propria and the draining lymph nodes (Kydd et al., 1994; Gryspeerdt et al., 2010; Vandekerckhove et al., 2010; Dunowska, 2014). So far, the effect of an EHV-1 infection of epithelial cells on the underlying basement membrane is unknown.

In the present study a nasal mucosal explant model was used to evaluate the effect of EHV-1 infection of epithelial cells on the integrity of the basement membrane. It has already been shown that EHV-1 spreads horizontally in a plaquewise manner in the respiratory epithelium (Gryspeerdt et al., 2010; Vandekerckhove et al., 2010). In contrast to SHV-1, BoHV-1 and HSV-1, EHV-1-induced plaques cannot breach the basement membrane barrier (Glorieux et al., 2007; Glorieux et al., 2011; Steukers et al., 2012, Vandekerckhove et al., 2010). However it penetrates in a more discrete manner, by exploiting individual monocytic cells and T-lymphocytes, and using them as a Trojan horse (Vandekerckhove et al., 2010; Bannazadeh Baghi and Nauwynck, 2014). While no generally accepted model of immune cell transmigration through the mucosal basement membrane exists, some potential mechanisms have been proposed: transmigration of cells through preformed holes or incisions, proteolytic digestion of extracellular matrix components and non-proteolytic force-dependent mechanisms (Rowe and Weiss, 2008; Sorokin, 2010). To determine if some components of the basement membrane are affected during EHV-1 infection the concentration and localization of integrin alpha 6, laminin, collagen IV and collagen VII under the plaques were investigated via quantitative image analysis.

Integrins are cell-surface molecules that play a role in cell-to-cell, cell-to-matrix adhesion and cell migration mechanisms (Mercurio, 1995; Wang et al., 2005). They have been identified on many different cell types, markedly in cells of an epithelial nature which interact strongly with fibronectin and laminin in the basement membrane (Kajiji et al., 1989; Müller et al., 2008). Considerable evidence exists that some integrins such as integrin alpha 6 beta 4 play a functional role in the hemidesmosomal anchoring complex (Liebert et al., 1994; Borradori and Sonnenberg, 1999). It is also well-known that during inflammation, activated integrins firmly bind VCAM-1 and ICAM-1 to stabilize adhesion and support leukocyte migration (McGettrick et al., 2012). However, little is known about the relative expression, distribution or function of different integrins on epithelial cells as well as in leukocyte subpopulations and endothelial cells. In the present study it was shown that integrin alpha 6 (ITGA6) completely disappeared underneath the EHV-1-infected zone. It is tempting to speculate that EHV-1-triggered degradation of integrin alpha 6 on the mucosal surfaces may facilitate transmigration of infected monocytic cells and T-lymphocytes through the mucosal basement membrane barrier en route to the blood vessels of the lamina propria and the draining lymph nodes. Alteration of integrin alpha 6 during viral infection suggests that this integrin has more than one function: an anchoring function in normal resting mucosal epithelial cells, and a second function in effecting the motility of leukocyte cells during infection. It is known that some microorganisms degrade basement membrane components with their secretory or surface-bound proteases, or using ‘hijacked’ host proteases (such as plasminogen), during inflammatory responses resulting in increased tissue damage. During infection, partially degraded and exposed basement membrane components are attractive targets for adherence of pathogens, which can be achieved by various microbial surface-exposed adhesive proteins (Vanlaere and Libert, 2009).

The basic framework of the basement membrane is thought to be created by two independent and distinct networks of laminin and type IV collagen (Yurchenco and Patton, 2009; Mestres et al., 2014). Infection of mucosal epithelial cells with EHV-1 did not change the structure of laminin and collagen IV under the plaques. Since laminin and collagen type IV serve as the main branched structural components of the mucosal barrier, this suggests that the main barrier function of the basement membrane may remain intact during EHV-1 infection. One of the interesting results in this study was the increased thickness of the collagen VII under the EHV-1 plaques. Type VII collagen is a major component of the anchoring fibrils network of the mucosal basement

membrane that attaches the epithelium to the extracellular matrix (Osawa et al., 2000; Evans et al., 2010a). Previous studies addressing interactions between collagen VII and unpolymerized basement membrane molecules demonstrated that the amino-terminal, non-collagen-like domain 1 (NC1) of collagen VII interacts with laminin 5 and collagen IV (Chen et al., 1997; Rousselle et al., 1997). Other studies reported direct but weak interactions between collagen VII and collagen I (Brittingham et al., 2006; Villone et al., 2008). Liebert and colleagues also showed co-localisation of collagen VII with integrin alpha 6 beta 4 (Liebert et al., 1994). It is possible that in equine nasal mucosa, there is a direct interaction between collagen VII and integrin alpha 6. Since we showed that EHV-1 infection leads to integrin alpha 6 degradation, this may lead to impaired collagen-integrin interactions. Degradation of integrin alpha 6 during EHV-1 infection may then disturb the anchoring of collagen VII which, although speculative at this point, in turn may then lead to the observed swelling of collagen VII. It will be interesting to further investigate whether the swelling of collagen VII in the absence of integrin alpha 6 during EHV-1 infection of mucosal epithelium, may activate migration of mononuclear cells in the underlying tissues, since such migrating mononuclear cells may be hijacked by EHV-1 during passage of infected region.

In conclusion, the study delivers new insights on the presence and localization of different components of mucosal basement membrane molecules in normal and EHV-1-infected tissues. We found that EHV-1 infection has a major effect on two important basement membrane components, namely integrin alpha 6 and collagen VII. The interesting aspect of this finding was that they were inversely affected: while integrin alpha 6 was degraded by infection, the collagen VII layer increased in thickness during infection. It will therefore be interesting to examine whether modulation of these particular proteins plays an important role in the migration of the virus through the basement membrane.

Conflict of interest statement

The authors declare no conflicts of interest.

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CHAPTER V

GENERAL DISCUSSION

Outline

Mucosal surfaces of the respiratory tract are the primary site of replication of countless viruses and bacteria. These areas are under rigorous control of professional antigen presenting cells (APCs), such as dendritic cells (DCs) and monocytes/macrophages. In addition to these, mucosal lymphocytes, and even epithelial cells play important roles in modulating immune responses to incoming pathogens.

During a respiratory tract infection, the number of APCs, notably DCs, increase in the mucosal tissues by chemotactic influx of precursors that originate primarily from circulating monocytes (Auffray et al., 2007; Geissmann, 2007). Migration of these monocytic cells to non-lymphoid peripheral tissues such as the nasal mucosa and lungs, is mediated by so called ‘‘inflammatory’’ chemokines. Exposure of these cells to antigens in peripheral tissues initiates cell maturation (Steinman, 1991). During maturation, the monocytic cells increase the surface expression of co-stimulatory molecules such as CD38, CD40, CD80 CD83, and CD86 (Reis and Sousa, 2006). They also change their expression of cell surface chemokine receptors (Alvarez et al., 2008). One of the important roles of monocytic cells at the mucosal sites is to initiate and regulate innate immune responses, acquire foreign antigens, and present antigens in mucosa-associated lymphoid tissues (MALT) such as NALT (nasopharynx- or nose-associated lymphoid tissue), LALT (larynx-associated lymphoid tissue) and BALT (bronchus-associated lymphoid tissue) (Długońska and Grzybowski, 2012; Soloff and Barratt-Boyes, 2010).

Some pathogens, particularly viruses, are specialized in infecting migrating mucosal monocytic cells during their passage within and outside the mucosa. The respiratory tract is the main port of entry for equine herpesvirus type 1 (EHV-1). After primary replication in the epithelial cells of the upper respiratory mucosa, EHV-1 crosses the basement membrane barrier by hijacking mucosal leukocytes, mainly monocytic cells and T lymphocytes, and spreads in the underlying lamina propria, reaching blood vessels and lymphatic vessels (Gryspeerdt et al., 2010; Vandekerckhove et al., 2010). Via the blood circulation, EHV-1-infected leukocytes disseminate to important internal organs such as the pregnant uterus, central nervous system (CNS) and lymphoid organs (Gryspeerdt et al., 2010; Patel and Heldens, 2005). How EHV-1-infected leukocytes invade the mucosa and ultimately reach the target organs is still not elucidated. Unraveling the mechanism of transmigration of EHV-1-infected mononuclear cells throughout the epithelium and basement

membrane barrier of the mucosa and submucosa may open new strategies to interfere with the invasion of this alphaherpesvirus and manage the disease.

Inspired by this knowledge, mucosal monocytic cells in the nasal mucosa were characterized (Chapter 3.1) and their migration pattern was compared with those of blood-derived monocytic cells and monocyte-derived DCs in nasal mucosal explants. Afterwards, the impact of EHV-1 infection on the migration of these monocytic cells through equine nasal mucosa was investigated (Chapter 3.2). The data presented here also revealed the impact of EHV-1 on integrin alpha 6 and different components of the basement membrane in the nasal mucosa: laminin, collagen IV and collagen VII (Chapter 4).

Monocytic lineage cells

The isolation of equine nasal mucosal mononuclear phagocytes and their characterization had not been reported yet. In the present PhD thesis, a new technique was described to isolate equine nasal mucosal monocytic cells ($CD172a^+$ cells). Morphological examination showed that large cells extended their dendrites onto the plastic wells after 24 hours of culture. The cells were spherical in the beginning and became triangular with typically vacuoles and prominent pseudopodia after three days. From three till four days they were crawling more actively using dendrites and pseudopodia. After five and six days, the majority of the isolated cells firmly attached to the culture dish. The functional and phenotypical characterization of these monocytic lineage cells revealed that the isolated population consists of mononuclear phagocytic cells, notably immature DC.

The morphology of tissue resident macrophages is heterogeneous in terms of their function and phenotype (Gordon and Taylor, 2005; Wynn et al., 2013). They express a vast majority of sensing molecules, including scavenger receptors, nuclear hormone receptors, pattern recognition receptors, and cytokine receptors, which allow macrophages to monitor tissue microenvironments and act as pivotal cells for controlling infection and tissue damage (Geissmann et al., 2010a; Okabe and Medzhitov, 2014). Monocyte-derived DCs have already been generated in various species, including: pigs, cattle, sheep, dogs, cats, and horses (Howard et al., 1999; Paillot et al., 2001; Chan et al., 2002; Bienzle et al., 2003; Mauel et al., 2006; Wang et al., 2007). Cultivation and characterization of equine monocyte-derived DCs, in terms of morphology and function, have been largely studied (Siedek et al., 1997; Mauel et al., 2006; Dietze et al., 2008; Cavatorta et al., 2009).

However, the isolation and characterization of mucosal mononuclear phagocytes, such as mucosal DCs and macrophages were not yet performed in most animals, including horses.

The resemblance of equine nasal mucosal CD172a$^+$ cells (nmCD172a$^+$ cells) to immature dendritic cells

The upper respiratory tract of horses is an important site for invading pathogens (e.g. viruses such as EHV-1 and EAV). As a consequence it plays a crucial role in host defense via the mucosal immune response. To this end, local monocytic cells such as macrophages and various DC subsets are believed to play a key role in the ablility to respond vigorously to microbial pathogens (Banchereau and Steinman, 1998).

In the present PhD research, preparation of single cell suspension from equine nasal tissues was done by collagenase type IV and DNase I. Subsequently antibodies against CD172a were used for isolation of mucosal monocytic lineage using MACS. We evaluated the morphology, phenotype and function of equine nasal mucosal CD172a$^+$ cells and compared them with blood-derived monocytes and blood monocyte-derived DCs. From the morphological point of view, cytoplasmic extrusions and diverse branched protoplasmic extensions were observed in the isolated nasal mucosal CD172a$^+$ cells. The triangular isolated monocytic cells, showed a typical dendritic morphology. The key morphological characteristic of DCs is the presence of countless membrane processes that extend out from the main cell body (similar to dendrites on neurons) (Steinman and Cohn, 1973; Merad et al., 2008). CD1c, CD83, CD172a, CD206 and MHC II were used to characterize equine nasal mucosal CD172a$^+$ cells. The expression of CD1c$^+$ cells was significantly higher in isolated CD172a$^+$ cells from the nasal mucosa compared to blood-derived monocyte and monocyte-derived DCs. This is in line with the result of isolated mucosal DCs from the epithelium and lamina propria of the human respiratory tract (Jahnsen et al., 2004). The expression of CD83, which is one of the widely known maturation markers on human and murine DCs on nasal mucosal CD172a$^+$ cells, was slightly higher than on blood-derived monocytes and monocyte-derived DCs. Due to the slightly higher expression of CD83 in the nasal mucosal CD172a$^+$ cells it is tempting to speculate that they are semi-mature cells in the mucosal area. These results correlate with the expression of the mannose receptor CD206 in both immature blood monocyte derived DCs and isolated nasal mucosal CD172a$^+$ cells. The low expression of MHC class II on the isolated cells, is another indication that these cells are rather immature cells. It is also well-documented that

immature DCs are able to take up antigens, via receptor-mediated endocytosis and macropinocytosis, more than their mature counterparts. The DC maturation causes down-regulation of the antigen-uptake machinery, up-regulation of costimulatory and adhesion molecules, maturation marker CD83, and peptide-MHC complexes (Banchereau and Steinman, 1998; Berchtold et al., 1999). In our study, the freshly isolated nasal mucosal CD172a$^+$ cells showed a high phagocytic activity. These findings are in line with the reported strong endocytic activity of monocytic cells in the human upper respiratory mucosa (Von Garnier et al., 2005).

Hence, the isolated nasal mucosal CD172a$^+$ cells resemble immature DCs based on morphology, function, and stage of differentiation. A better understanding of nasal mucosal mononuclear immune cells could shed new light on how viruses (such as arteriviruses and alphaherpesviruses) may hijack these crucially important cells during invasion and immune evasion in the mucosal area and how they may modulate the immune response against secondary infections with other viral and bacterial pathogens.

Modulation of monocytic cell migration by viral infection

Monocytic cells represent important cellular targets for many viruses. They can enter in lymphoid tissues during inflammation and give rise to macrophages and inflammatory DCs (Gordon and Taylor, 2005; Luster et al., 2005; Geissmann et al., 2010a). These mononuclear phagocytes are considered to be an important cellular target for some viruses such as human immunodeficiency virus type-1 (HIV-1) and EHV-1 (Haase, 2010; Gryspeerdt et al., 2010; Vandekerckhove et al., 2010).

One of the goals of this doctoral research (Chapter 3, part B) was to identify and to decipher new immune evasion mechanisms of EHV-1, focusing on the different types of monocytic cell transmigration in the nasal mucosal tissue. We designed a system to evaluate the migration of different types of monocytic cells in the upper respiratory tract and to determine their importance for EHV-1 transmigration. The explant model allowed us to study the movement of non-infected monocytic cells and the transmigration of EHV-1-infected cells within nasal mucosa. This *in vitro* model of nasal mucosal epithelium is a valuable system to provide novel information on the early pathogenesis of not only EHV-1 but also other animal and human viruses. Indeed, behavior of the transmigration of these mononuclear cells alone and virus-infected cells in the *in vitro* explant model is highly reminiscent of the *in vivo* situation in the natural hosts.

A percentage of non-infected monocytic cells (16-26%) invaded the mucosa between 24 and 48 hours after the cells were added on top of the epithelium. We could thereby show that blood-derived monocytes did not migrate into deep layers of the lamina propria and submucosa in our *in vitro* system. However, we demonstrated that monocyte-derived DCs and nasal mucosal CD172a$^+$ cells act as potential transportation vehicles, by migration into deep mucosal tissue (1-7%). These observations agree with the well accepted migratory behavior of mucosal macrophages and DCs, which breach the basement membrane barriers, in order to be able to migrate to draining lymph nodes and present antigens to naïve T cells (Kissenpfennig et al., 2005; Coombes and Powrie, 2008; Tugizov et al., 2012). Monocytic cells migrate from blood vessels towards regions of inflammation. However it is not mentioned that they also migrate back. Therefore nasal macrophages and DCs are all ideal vehicles for pathogens to enter its host at the respiratory mucosa.

With the help of the established explant model, we evaluated the migration pattern of three EHV-1-inoculated monocytic cell types. Our results indicated that both EHV-1-inoculated and mock-inoculated monocytic cells migrated into the epithelium and lamina propria to a similar extent.Similarly to mock-inoculated cells, the number of EHV-1-infected monocyte-derived DCs and nasal mucosal mCD172a$^+$ cells that passed throughout the nasal epithelium was significantly higher than EHV-1-infected blood-derived monocytes after 48 hours. Despite the fact that all types of monocytic cells were able to capture EHV-1 from the mucosal surface (which results in the infection of the cells) and to penetrate into mucosal epithelia, only monocyte-derived DCs could transfer EHV-1 through the basement membrane barrier at 24 hours. After 48 hours, nasal mucosal CD172a$^+$ cells were able to carry EHV-1 through the basement membrane barrier but to a much lesser extent than monocyte-derived DCs. Blood-derived monocytes took up EHV-1, but failed to breach the basement membrane barrier. This may be due to the inability of these cells to differentiate into antigen presenting cells (Palframan et al., 2001; Jakubzick et al., 2013). Considerable evidence indicates that mononuclear cells, notably DCs and macrophages, are actively involved in the transmigration of pathogens from mucosal surfaces towards local lymph nodes. Our results are consistent with the previous observations on the transmigration of HIV-infected mononuclear cells through the paracellular space of intact mucosal epithelia and paracellular space of polarized endometrial epithelial cells (Carreno et al., 2002; Anderson et al., 2010; Tugizov et al., 2012).

In conclusion, we found that while EHV-1 can infect three types of monocytic cells to a varying extent, the virus did not obviously change the migratory behavior of the cells in a positive or negative way. Moreover, we could show that monocyte derived dendritic cells and nasal mucosal $CD172a^+$ cells are able to carry EHV-1 from the surface of nasal mucosa to the deep lamina propria and submucosa, en route to the lymph and blood circulation. These data suggest that these monocytic cell types may play a pivotal role in the early stages of EHV-1 infection.

Role of basement membrane barrier in EHV-1 invasion

In Chapter 4 the invasive effect of EHV-1 infection (neurovirulent strain, 03P37) on different components of the basement membrane was evaluated. Components of the basement membranes, integrin alpha 6, laminin, collagen IV and collagen VII, function as mechanical containment molecules that protect tissues against infection of pathogens. The presence of these different components in normal and EHV-1-infected nasal mucoal tissues was investigated by measuring their thickness with the software imaging system ImageJ.

Little is known about the transmigration and penetration of alphaherpesviruses from the apical side of mucosal epithelium towards the basolateral side. Previous work, in our laboratory, revealed that PRV breaches the basement membrane plaquewise after epithelial infection, involving protease-mediated (trypsin-like serine protease) disintegration of the extracellular matrix, in order to invade lamina propria and distribute in the body (Glorieux et al., 2011). In contrast, EHV-1 does not cross the basement membrane in a plaquewise manner but instead infects resident monocytic cells within the respiratory epithelium and appears to abuse their motile nature to invade the host (Vandekerckhove et al., 2010). The involvement of different components of the extracellular matrix and notably the basement membrane barrier in the invasion process of alphaherpesviruses remains poorly understood.

Populations of leukocytes constantly travel through basement membrane barriers to patrol host tissues in their search for microbial pathogens (Ley et al., 2007). During pathologic events such as neoplastic events and microbial infection, cells can misuse normal basement membrane transmigration programs. To this end, the basement membrane pore size needs to be transformed. This happens mainly through protease-dependent disintegration and is largely reliant on matrix metalloproteinases (MMP) (Sherwood, 2006; Kelley et al., 2014). Indeed, leukocytes can traffic across the basement membrane barrier, without leaving detectable perforations. However, the

exact mechanism of both physiologic and pathologic cell transmigration remains complex and, to a great extent, unresolved. Therefore, further studies into the transmigration of immune cells from the basement membrane barrier are needed. In this thesis, four proteins of extracellular matrix (integrin alpha 6, laminin, collagen IV and collagen VII), which are mainly associated with the basement membrane barrier, were assessed. It goes without saying that all mucosal-invasive viruses as well as free EHV-1 and EHV-1-infected cells have to overcome and breach this obstructive membrane. This will help further invasion of viruses into underlying lamina propria and ultimately into their end target organs. Here we could demonstrate that an EHV-1 infection clearly affects some components of the nasal mucosal barrier and the virus degrades integrin alpha 6.

Integrins are cell-surface molecules that play a role in cell-to-cell adhesion, cell migration mechanisms and act as ligands of basement membrane components (Mercurio, 1995; Wang et al., 2005). Relative expression, distribution or function of different integrins on epithelial cells as well as in leukocyte subpopulations and endothelial cells remains a highly undiscovered field. Since integrin alpha 6 is disintegrated so strongly during an EHV-1 infection, it is interesting to look further into its mechanism and to examine if it is helping monocytic cells to find their way to the infected epithelial cells. Elucidating this mechanism could bring new insights on how to block it. Research should focus on which viral components are modulating this process. In this context, mutants can shed new light on the mechanism. Deleting the viral genes that are encoding proteins that are responsible for the integrin alpha 6 disintegration may help to attenuate EHV-1 and to develop vaccines. Because EHV-4, which is closely related to EHV-1, is much less pathogenic because it does not invade via leukocytes, it would be worth looking at its interaction with integrin alpha 6. Some microorganisms degrade extracellular matrix components with their secretory or surface-bound proteases, or ‘hijack’ host proteases. As mentioned above, the present study shows a disintegration of integrin alpha 6 underneath EHV-1 infected regions. With this knowledge, it may be questioned whether this phenomenon is responsible for the transmigration of infected monocytic cells and T-lymphocytes throughout the mucosal basement membrane barrier en route to the blood vessels of the lamina propria and the draining lymph nodes. Another finding in this study was that while integrin alpha 6 was disintegrated, collagen VII under the EHV-1 plaques increased in thickness. It is possible that there is a direct interaction between collagen VII and integrin alpha 6 in the equine nasal mucosa. Damage of integrin alpha 6-collagen VII interaction

may allow the collagen VII layer to swell. Another possibility is that the production of collagen VII is activated due to EHV1 infection, leading to a thicker layer. It should be noted that EHV-1-infected cells and free viruses did not affect the thickness of laminin and collagen IV in the mucosal area.

Based on the results from the present PhD thesis, the following hypothetical pathogenesis model may be forwarded as illustrated in Figure 1. During an EHV-1 infection of epithelial cells, integrin alpha 6 becomes disintegrated. The disconnection of the bonds between epithelial cells and collagen VII leads to the loosening of the infected epithelial cells and swelling of collagen VII. These structural changes are attracting monocytic cells that are guided in the direction of infected epithelial cells. Afterwards, the monocytic cells become infected and move in a physiological way back to lymph and blood vessels.

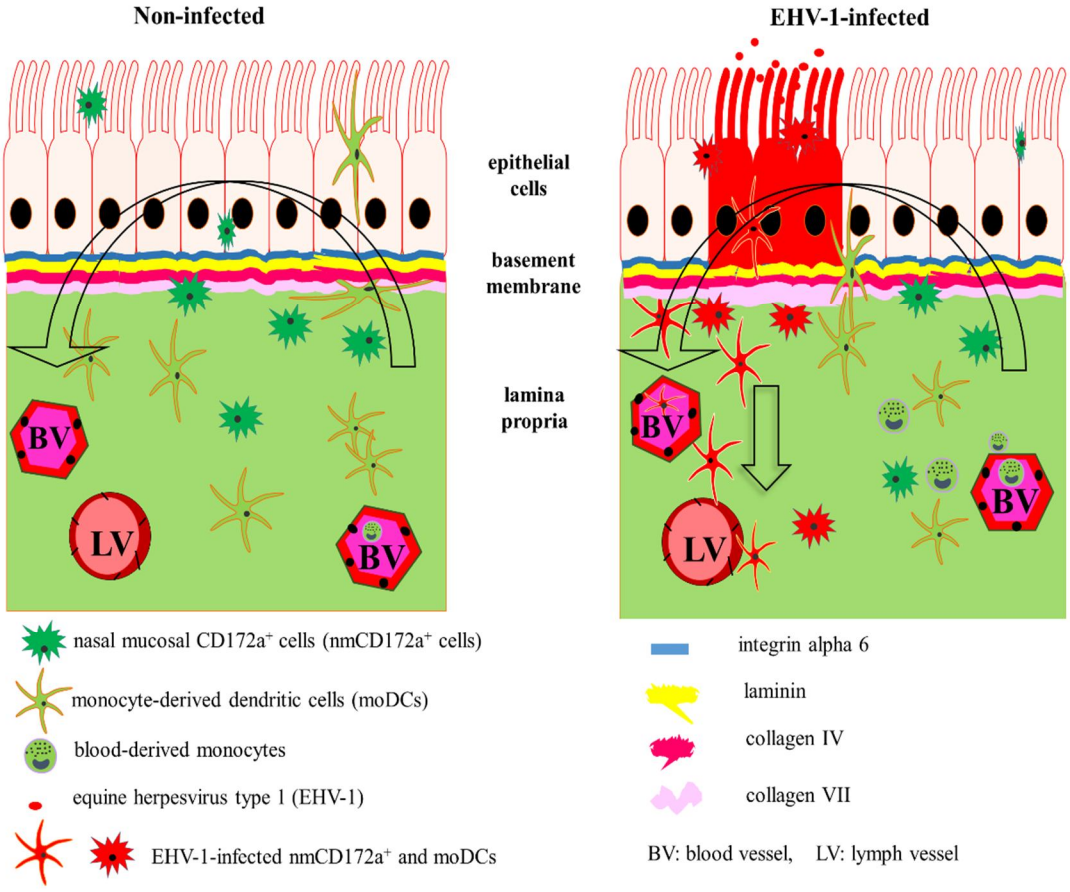

Figure 1. *Hypothetical explanation for the invasion mechanism of EHV-1-infected mononuclear cells through the basement membrane.*

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CHAPTER VI

SUMMARY – SAMENVATTING

Summary

Equine herpesvirus type 1 (EHV-1) is an alphaherpesvirus causing respiratory problems, abortion, neonatal foal death, chorioretinopathy and equine herpesvirus myeloencephalopathy (EHM). The upper respiratory tract (especially nasal mucosa) is the primary site of virus replication. After local replication, EHV-1 spreads via a cell-associated viremia reaching the internal target organs such as uterus and central nervous system. Replication in endothelial cells of these organs can result in abortion or nervous system disorders. Peripheral blood mononuclear cells (PBMC), notably monocytic cells and T-lymphocytes, play a key role in the pathogenesis of an EHV-1 infection, both in naïve and in immune horses. These immune cells perform a crucial role in transporting EHV-1 from the primary site of replication to the target organs. However, the lack of knowledge about the invasion strategies of immune EHV-1-infected cells is an important obstacle.

Chapter 1, is divided into two sections. First, an introduction is given about EHV-1 in general, describing its phylogenetic background, taxonomy and virus structure, genomic organization and replication cycle, pathogenesis, latency and symptoms. Second, an overview of cell migration is given, with a focus on immune cell transmigration including leukocytes trafficking, recirculation of leukocyte, leukocyte rolling and activation during tethering, integrins in leukocyte migration, leukocyte recruitment in inflammation and the role of ICAM-1 and VCAM-1 in leukocyte adhesion.

In **Chapter 2**, the general goals of the study described in the present dissertation were put forward. The aims include the isolation and characterization of nasal mucosal monocytic cells from the upper respiratory mucosa. Then, the migration pattern of the isolated monocytic cells and two blood-derived cells (blood-derived monocytes and blood monocyte-derived DCs) in nasal mucosal explants were examined together with the effect of EHV-1 infection on the migratory behavior of these cells. Finally, the effect of EHV-1 infection on different components of the basement membrane were evaluated.

In **Chapter 3 A**, a detailed characterization of monocytic cells ($CD172a^+$ cells), residing in the equine nasal mucosa was performed from five horses. Nasal mucosal $CD172a^+$ cells were isolated from collagenase type IV and DNase I digested equine nasal mucosa fragments by magnetic activated cell sorting. Equine blood-derived monocytes and blood monocyte derived dendritic cells (moDCs) were isolated and generated to compare them with the isolated mucosal cells. Surface markers of all three cell types were determined by flow cytometry. The expression of cell surface markers in the nasal mucosal cells was as follows: 90% $CD172a^+$, 30% $CD1c^+$, 46% $CD83^+$, 42% $CD206^+$ and 28% MHC II^+. In blood-derived monocytes: 96% $CD172a^+$, 4% $CD1c^+$, 11% $CD83^+$, 9% $CD206^+$, 72% MHC II^+ and on blood monocyte derived DCs: 99% $CD172a^+$, 13% $CD1c^+$, 30% $CD83^+$, 51% $CD206^+$ and 93% MHC II^+. Functional analysis of the cells was done by measuring the uptake of FITC conjugated ovalbumin (FITC-OVA). The $CD172a^+$ nasal mucosal cells were functionally able to endocytose FITC-OVA but to a lesser degree than monocyte-derived DCs. These results demonstrated that the isolated $CD172a^+$ nasal mucosal cells resemble immature DCs in the nasal area.

In **Chapter 3 B**, we aimed to evaluate how EHV-1 effects the migratory behaviors of monocytic cells in the mucosal explant model. EHV-1 replicates extensively in the epithelium of the upper respiratory tract, after which it can easily spread throughout the body via a cell-associated viremia in mononuclear leukocytes (mostly monocytic cells and T lymphocytes) reaching the pregnant uterus and central nervous system. In Chapter 3 A, three monocytic cell types were isolated and characterized. These monocytic cells were labeled with a fluorescent dye (CFSE) and were transferred to the apical part of a polarized mucosal explant system. We monitored the migration pattern of monocytic cells and the effect of EHV-1 on transmigration of these cells by means of confocal microscopy at different time points. We could show that one-fourth of both EHV-1-inoculated and mock-inoculated monocyte-derived DCs and nasal mucosal $CD172a^+$ cells moved into the nasal epithelium. A fraction moved even further into the lamina propria and submucosa. Virus-inoculated blood-derived monocytes did not move through the basement membrane barrier. In general we could conclude that nasal mucosal $CD172a^+$ cells and monocyte derived DCs are able to transport EHV-1 into the submucosa which facilitates a generalized infection with the virus.

In **Chapter 4**, the aim was to determine the impact of an infection with EHV-1 on different components of the basement membrane. Since an important prerequisite for successful host invasion of the virus is to cross the epithelial cell layer and the underlying basement membrane barrier, the focus of the analysis was the possible damaging of the underlying basement membrane by the EHV-1 infection. In this context, a detailed quantitative analysis was set up which allowed an in-depth analysis of thickness of areas stained for integrin alpha 6, laminin, collagen IV and collagen VII under the virus positive plaques in comparison with the thickness of the membrane in the non-infected areas. The results indicated that (i) integrin alpha 6 disappeared under the EHV-1 plaques, (ii) the thickness of collagen VII increased and (iii) the thickness of laminin and collagen IV stayed unaffected compared to mock-inoculated tissues. The results thus revealed that integrin alpha 6 and collagen VII are highly involved in the EHV-1 invasion process.

In **Chapter 5**, the main findings of this dissertation are discussed. Monocytic cells do play an important role in the transmigration process of EHV-1 through the mucosal area, the basement membrane and the further spread of the virus throughout the body. Their patrolling functions allow the virus to hijack them and use them as a transportation means into the tissue. We discussed the isolation and characterization of nasal mucosal $CD172a^{+}$ cells and used blood monocytes and blood-derived moDCs as controls. It was found that while nasal monocytic cells originally are meant to fulfill a preventive function against infection in the nasal mucosal epithelium, they become the very means by which the virus is spread throughout the body. Here it was clearly shown that the consequences of EHV-1 infection of nasal mucosa are the degradation of integrin alpha 6 and the thickening of collagen VII. This interplay of these basement membrane components facilitates the transmigration of infected monocytic cells into the tissue.

Samenvatting

Equiene herpesvirus type 1 (EHV-1) is een alfa-herpesvirus dat ademshalingsproblemen, spontane abortus, neonatale veulen sterfte, chorioretinopathie en equiene herpesvirus myeloencephalopathie (EHM) veroorzaakt. De primaire locatie van virus replicatie is het neusslijmvlies van de bovenste ademhalingswegen. Na de lokale replicatie, verspreidt EHV-1 zich via een cel-geassocieerde viremia en bereikt hiermee de interne doelorganen, zoals de baarmoeder en het centrale zenuwstelsel. Virale replicatie in de endotheliale cellen van deze organen kan in een spontane abortus of zenuwstelsel aandoeningen resulteren. Perifere bloed mononucleaire cellen (PBMC), met name monocyten en T-lymfocyten, spelen een belangrijke rol bij de pathogenese van een EHV-1 infectie, zowel in naïeve als in immune paarden. Deze immuuncellen vervullen een cruciale rol bij het transport van EHV-1 uit de primaire plaats van replicatie naar de doelorganen. Echter, het gebrek aan kennis over de invasie strategieën van EHV-1-geïnfecteerde immuuncellen is een belangrijk obstakel.

Hoofdstuk 1 is verdeeld in twee secties. Eerst wordt er een inleiding gegeven over EHV-1 in het algemeen, met een beschrijving van de fylogenetische achtergrond, taxonomie en virusstructuur, genomische organisatie en replicatiecyclus, pathogenese en symptomen. Ten tweede wordt er een overzicht gegeven van de cel migratie, met een nadruk op immuuncel transmigratie waaronder verplaatsing, recirculatie, rollen en activering tijdens migratie van leukocyten, leukocytaantrekking bij ontstekingen en de rol van ICAM-1 en VCAM-1 in leukocytenadhesie.

In **Hoofdstuk 2** worden de algemene doelstellingen van het in dit proefschrift beschreven onderzoek naar voren gebracht. De doelstellingen omvatten de isolatie en karakterisering van nasale mucosale monocytische cellen van het bovenste luchtwegen slijmvlies. Ook het migratiepatroon van de geïsoleerde monocytische cellen en twee bloed afgeleide cellen (bloed monocyten en bloed-monocyt-afgeleide DCs) in nasale mucosale explantaten samen met het effect van EHV-1 infectie op het migratiegedrag van deze cellen vormt een belangrijke doelstelling. Een laatste doelstelling tenslotte omvat het nagaan van het effect van EHV-1 infectie op verschillende componenten van het basale membraan.

In **Hoofdstuk 3 A**, werd er een gedetailleerde karakterisatie van monocytische cellen ($CD172a^+$ cellen), welke aanwezig zijn in het equiene neusslijmvlies, uitgevoerd bij vijf paarden. Nasale mucosale $CD172a^+$ cellen werden geïsoleerd, uit de door collagenase type IV en DNase I afgebroken equiene neusslijmvlies fragmenten, door middel van magnetisch geactiveerde celsortering. Equiene bloed monocyten en van bloed monocyten afgeleide dendritische cellen (moDCs) werden geïsoleerd en gegenereerd om te kunnen vergelijken met de geïsoleerde mucosale cellen. Oppervlaktemarkers van alle drie de celtypes werden bepaald door flowcytometrie. De expressie van celoppervlakmarkers in de nasale mucosale cellen was als volgt: 90% $CD172a^+$ 30%, $CD1c^+$, 46% $CD83^+$, $CD206^+$ 42% en 28% MHC II^+. In bloed monocyten: 96% $CD172a^+$, 4% $CD1c^+$, 11% $CD83^+$, 9% $CD206^+$, 72% MHC II^+ en op van bloed monocyten afgeleide DC's: 99% $CD172a^+$, 13% $CD1c^+$, 30% $CD83^+$, 51% $CD206^+$ en 93 % MHC II^+. Functionele analyse van de cellen werd uitgevoerd door meting van de opname van FITC geconjugeerd ovalbumine (OVA-FITC). De $CD172a^+$ nasale mucosale cellen konden actief FITC-OVA endocytoseren maar in mindere mate dan monocyt afgeleide DCs. Deze resultaten toonden daarmee aan dat de geïsoleerde $CD172a^+$ nasale mucosale cellen op onrijpe DCs lijken.

In **Hoofdstuk 3 B**, hebben we geëvalueerd hoe EHV-1 het migratie gedrag van monocytcellen in de mucosale explantaatmodel beïnvloedt. EHV-1 repliceert excessief in het epitheelweefsel van de bovenste luchtwegen, waarna het zich gemakkelijk kan verspreiden in het lichaam via een celgebonden viremie in mononucleaire leukocyten (meestal monocytische cellen en T-lymfocyten) met als laatste bestemming de drachtige baarmoeder en het centrale zenuwstelsel. In hoofdstuk 3 A werden drie monocytaire celtypen geïsoleerd en gekarakteriseerd. Deze monocytische cellen werden gemerkt met een fluorescente kleurstof (CFSE) en overgebracht naar het apicale deel van een gepolariseerde mucosale explantaat systeem. We volgden het migratiepatroon van monocytische cellen en het effect van EHV-1 op transmigratie van deze cellen door middel van confocale microscopie op verschillende tijdstippen. Wij konden aantonen dat één vierde van zowel de EHV-1 geïnoculeerde als de mock-geïnoculeerde monocyt afgeleide DCs en nasale mucosale $CD172a^+$ cellen zich verplaatst hadden naar het nasale epitheel. Een fractie had zich nog verder verplaatst naar de lamina propria en submucosa. Virus geïnoculeerde bloed monocyten waren niet door het basaal membraan barrière gemigreerd. In het algemeen kunnen we

dus concluderen dat nasale mucosale CD172a^{+} cellen en van bloed monocyten afgeleide DCs EHV-1 kunnen transporteren naar de submucosa, wat een algemene infectie met het virus vergemakkelijkt.

In **Hoofdstuk 4**, was het het doel om de gevolgen van een infectie met EHV-1 op de verschillende onderdelen van het basaalmembraan te bepalen, aangezien een belangrijke voorwaarde voor een succesvolle gastheerinvasie van het virus erin bestaat dat het virus de epitheliale cellaag en onderliggende basaalmembraan passeert. De focus van de analyse lag op de mogelijke beschadiging van het onderliggende basaalmembraan door de EHV-1 infectie. In dit verband werd een gedetailleerde kwantitatieve analyse op punt gesteld, die een grondige analyse van de dikte van gebieden gekleurd voor integrine alfa 6, laminine, collageen IV en collageen VII onder virus positieve plaques toestond ten opzichte van de dikte van het membraan in de niet-besmette gebieden. De resultaten gaven aan dat (i) integrine alfa 6 degradatie onderging onder de EHV-1 plaques, (ii) de dikte van de collageen VII laag toegenomen was en (iii) de dikte van de laminine en collageen IV lagen onveranderd bleven in vergelijking met de mock-geïnoculeerde weefsels. De resultaten onthulden hiermee dat integrine alfa 6 en collageen VII mogelijks betrokken zijn bij het EHV-1 invasie proces.

In **Hoofdstuk 5** worden de belangrijkste bevindingen van dit proefschrift besproken en bediscussieerd. Monocytische cellen spelen een belangrijke rol in het proces van EHV-1 transmigratie door het mucosale gebied, het basale membraan en de verdere verspreiding van het virus in het lichaam. Hun patrouillerende functies laten het virus toe om ze te kapen en ze als een transportmiddel te gebruiken naar de weefsels. Daarnaast wordt de resultaten besproken die aantoonden dat EHV-1 infectie van het neusslijmvlies, zowel de afbraak van integrine alfa 6 een verdikking van de collageen VII laag als gevolg heeft. De wisselwerking van deze membraancomponenten vergemakkelijkt mogelijks de transmigratie van geïnfecteerde monocytische cellen in het weefsel.

CURRICULUM VITAE

Hossein Bannazadeh Baghi was born in Tabriz, Iran. He completed his high school studies with a major in science in 1998. He received a BSc in Biomedical Sciences (a.k.a Medical Laboratory Sciences) from Tabriz Medical University in 2003 and an MSc in Medical Virology from Tarbiat Modares University, Tehran, Iran in 2006. He did his MSc thesis on the construction of vectors that express vhs from HSV-2 and 3Cpro from Coxsackievirus B3 and the comparison of their effect on eukaryotic cells.

Hossein has been a lecturer in Medical Virology in Tabriz Medical University and Medical Microbiology in Payame Noor University of Marand and has been involved in several research projects in both universities and at the Ministry of Health and Medical Education of Iran from 2006 till 2010.

From 2010 - 2015, he performed his PhD thesis in the Department of Virology, Parasitology and Immunology at the University of Gent. He investigated the effect of an EHV-1 infection on the migration behavior of monocytic cells and on components of the basement membrane in the respiratory mucosa.

Hossein is the author and coauthor of several scientific papers in peer-reviewed journals and has presented his research results in national and international meetings.

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PUBLICATIONS

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MEMBERSHIP

1. Belgian Society of Virology (BELVIR)
2. Iranian Society of Microbiology (No: 605)
3. Iranian Society for Virology (No: 1135)
4. Iranian Genetic Society (I.G.S) (No: 115)
5. Iranian Biotechnology Society (I.B.S) (No: 74)
6. Young Researchers Club (No: 872243350)

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Hossein
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"A man's worth depends upon the nobility of his aspirations."
"The sum total of excellence is knowledge"
~ Ali Ibn Abu-Talib (a.s.)